9B13 Scanned

#39 2/23/02 TBray

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Lars Abrahmsen et al.

Docket No.: HO-P01525US0

Application No.: 08/765,695

Group Art Unit: 1644

Filed: July 25, 1997

Examiner: R. Schwadron

For: A CONJUGATE BETWEEN A MODIFIED SUPERANTIGEN AND A TARGET-SEEKING COMPOUND AND THE USE OF THE CONJUGATE

TRANSMITTAL OF AMENDED APPELLANT'S BRIEF

Attention: Board of Patent Appeals and Interferences
Commissioner for Patents
Washington, DC 20231

Dear Sir:

Appellants hereby submit an original and two copies of this amended Appeal Brief to the Board of Patent Appeals and Interferences in response to notice of non-compliant Appeal Brief, which was mailed on January 3, 2001. Appellants are filing this amended Brief in accordance with 37 CFR § 1.192(d). Appellants assert that the amended Brief satisfies 37 CFR § 1.192(c). Appellants do not believe that there are any additional fees for the filing of this amended Appeal Brief. If however, Appellants are not correct, then the Appellants hereby authorize the Commissioner to deduct any fees for filing of the amended Appeal Brief from the Fulbright & Jaworski Deposit Account No. 06-2375, Order No. 09804877.

Dated: January 29, 2002

Respectfully submitted,

By _____
Melissa W. Acosta
Registration No.: 45,872
FULBRIGHT & JAWORSKI L.L.P.
1301 McKinney, Suite 5100
Houston, Texas 77010-3095
713-651-5407
713-651-5246 FAX

RECEIVED
FEB 0 1 2002
TECH CENTER 1600/2900

(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Lars Abrahmsen et al.

Docket No.: HO-P01525US0

Application No.: 08/765,695

Group Art Unit: 1644

Filed: July 25, 1997

Examiner: R. Schwadron

For: A CONJUGATE BETWEEN A MODIFIED SUPERANTIGEN AND A TARGET-SEEKING COMPOUND AND THE USE OF THE CONJUGATE

AMENDED APPELLANT'S BRIEF

TABLE OF CONTENTS

PAGE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Lars Abrahmsen

Application No.: 08/765,695

Group Art Unit: 1644

Filed: July 25, 1997

Examiner: R. Schwadron

For: A CONJUGATE BETWEEN A MODIFIED SUPERANTIGEN AND A TARGET-SEEKING COMPOUND AND THE USE OF THE CONJUGATE

AMENDED APPELLANT'S BRIEF

Attention: Board of Patent Appeals and Interferences
Commissioner for Patents
Washington, DC 20231

Dear Sir:

Appellants hereby submit an original and two copies of this Appeal Brief to the Board of Patent Appeals and Interferences in response to notice of non-compliant Appeal Brief, which was mailed on January 3, 2001. The original Appeal Brief was in filed in response to the Advisory Action dated July 24, 2001 and the final Office Action dated April 17, 2001. This brief is in furtherance of the Notice of Appeal, filed in this case on August 15, 2001.

I. REAL PARTY IN INTEREST

The real party in interest for this appeal is the assignee, Pharmacia & Upjohn AB.

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences, which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS

Claims 14-51 were originally filed in the present application. During prosecution, claims 52-57 were added, claims 14-35, 39-43 and 48-51 were canceled, claims 53-57 were withdrawn from consideration but not canceled and claims 36-38 and 44-47 were amended. The claims on appeal are claims 36-38, 44-47 and 52 of which a copy of the claims in what Appellants believe to be the correct status is attached as Appendix A.

IV. STATUS OF AMENDMENTS

Appellants filed an Amendment After Final, which was filed on June 25, 2001, to amend the Abstract. The Amendment to the Abstract was entered by the Examiner indicated by the Advisory Action, which was mailed on July 24, 2001.

V. SUMMARY OF INVENTION

The present invention uses a conjugate comprising a mutated superantigen to treat a disease or a condition in a mammal. The mutated superantigens are based upon Appellant's discovery that different regions of superantigens are responsible for binding the MHC class II antigens. Mutations in these regions can affect MHC class II binding resulting in decreased toxicity of the superantigens while retaining the therapeutic effects of the superantigens. For example, some of the therapeutic effects include activation of T lymphocytes which results in selective lysis of cells. *See* Specification p. 4, lns., 6-15; p. 9, lns., 18-23; Table III; and p. 22, lns., 4-10.

VI. ISSUES

The issues for the Board's consideration are:

Whether claims 36-38, 44-47 and 52 are properly rejected under 35 U.S.C. § 103(a) as being unpatentable over Dohlsten et al., PNAS USA 88:9287-9291(1991).

Whether the information disclosure statement complies with 37 CFR § 1.97(c).

VII. GROUPING OF CLAIMS

For purposes of this appeal brief only, the claims should stand or fall separately.

In Section VIII below, Appellants have included arguments supporting the separate patentability of each claim group as required by M.P.E.P. § 1206.

VIII. ARGUMENTS

A. Claim 36 is non-obvious.

The Action rejects claim 36 under 35 U.S.C. § 103(a) as being allegedly unpatentable over Dohlsten et al., PNAS USA 88:9287-9291, 1991. The Examiner contends that Dohlsten allegedly suggests making mutations in the C-terminal region of superantigens in order to reduce Class II MHC antigen binding. Appellants traverse this rejection.

i. Non-enabling Reference Is Not Available Prior Art

The Manual of Patent Examining Procedures (MPEP) sets forth the guidelines or conditions for patentability of non-obvious subject matter under 35 U.S.C § 103 in MPEP § 2141.01. This section clearly states that "before answering Graham's content inquiry, it must be known whether a patent or publication is in the prior art under 35 U.S.C. § 102. *Panduit Corp. v. Dennison Mfg. Co.*, 810 F.2d 1561, 1568, 1 USPQ2d 1593, 1597 (Fed. Cir.), cert. denied, 481 U.S. 1025 (1987). Both the Federal Circuit Court and the court of Customs and Patent Appeals have directed the Patent Office to determine if the prior art reference is available 35 U.S.C. § 102 as an enabling reference. See *In re Donohue*, 766 F.2d 531, 226 USPQ 619 (Fed Cir. 1985), *In re LeGrice*, 301 F.2d 939, 133 USPQ 365, 371 (CCPA 1962).

Appellants have clearly asserted that Dohlsten et al. is a non-enabling reference. Specifically, Dohlsten et al. do not teach or suggest making mutations in any particular region in order to affect class II MHC antigen binding. In addition, Appellants reiterate that the Office has acknowledged in the Office Action of August 16, 1999, page 5 that "Dohlsten et al. do not teach that the superantigen portion of the conjugate has been mutated to show a modified ability to bind class II MHC antigen".

Although the Office has acknowledged that Dohlsten et al. do not teach that the superantigen portion of the conjugate has been mutated to effect the binding of a Class II MHC antigen, the Office has continued to erroneously interpret statements in Dohlsten et al. The statements in the Dohlsten reference that the Office has erroneously interpreted are: i) "it would be of importance to further perturb MHC class II-dependent CTL activity by reducing the binding of the C215-SEA conjugate for MHC class II molecules" (page 9291, column 1) and ii) and "MHC class II binding has been localized to the C-terminal" (page 9291, column 1). These statements, taken alone or in combination, do not, in fact, teach that class II MHC binding is in the C-terminal region of superantigens. In fact, the next statement on page 9291, column 1 states that "determination of the amino acids necessary for MHC class II binding may provide a rationale to obtain mAb-SEA conjugates with preserve T-cell-activating properties by totally devoid of binding to MHC class II molecules". Thus, Dohlsten is clearly stating that it is not known what amino acids are required for MHC class II binding. Dohlsten et al. do not provide a guideline to determine which amino acids are necessary for binding. All of these statements clearly indicate that the reference is non-enabling for mutating superantigens to modify their ability to bind to MHC class II.

Further, the second statement, "MHC class II binding has been localized to the C-terminal" (page 9291, column 1), is, in fact, not correct when referring to the location for MHC Class II binding on SEC1 and toxin shock syndrome toxin 1. An immense body of

work has shown that the MHC class II binding region of SEB (structural homologue of SEC1) and TSST-1 resides in the N-terminal part of the protein and not in the C-terminal (*see* Kim et al., Science, 1994, 266:1870 (co-crystals of MHC class II antigens and TSST-1); and Jardetzky et al., Nature, 1994, 368:711 (SEB and MHC class II binding)).

Appellants assert that the present invention teaches for the first time the different regions of superantigens that are responsible for binding the MHC class II antigens and that mutations in these regions can affect MHC class II binding. *See* specification p. 23, lns., 12-15.

Appellants strongly contend that Dohlsten et al. is a non-enabling reference under 35 U.S.C. § 102 because it does not sufficiently describe the claimed invention to have placed the public in possession of it. *In re LeGrice*, 301 F.2d 939, 133 USPQ 365, 371 (CCPA 1962).

ii. Prima facie Case of Obviousness Must be Established

The Manual of Patent Examining Procedures (MPEP) sets forth the guidelines to establish a *prima facie* case of obviousness of non-obvious subject matter under 35 U.S.C § 103 in MPEP § 2143.3. This section clearly states that three basic criteria must be met to establish a *prima facie* case of obviousness. The three criteria include:

1) a suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings;

2) a reasonable expectation of success; and

3) the prior art reference must teach or suggest all the claim limitations.

In light of the above criteria, Appellants assert that the Office has not established a *prima facie* case of obviousness to reject the claims under 35 U.S.C. 103. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438, (Fed. Cir. 1991). A *prima facie* case necessitates disclosure of the source for either a suggestion or motivation to modify this reference to produce the present invention, and a reasonable expectation of success of producing the present invention. Dohlsten et al. do not provide a suggestion nor does it provide a reasonable expectation of success of producing a mutated superantigen having modified binding ability. This is clearly indicated by the acknowledgement of the Office that Dohlsten does not teach a mutated superantigen having a modified ability to bind to class II MHC antigens. (See Office Action dated August 16, 1999, p. 5)

Yet further, the court of Customs and Patent Appeals have directed the Patent Office to establish a *prima facie* case by evidence rather than conjecture. *Ex parte Yamamoto*, 575 USPQ2d 1382, 1383, 1384 (CCPA 2000). Appellants assert that the Office has not provided sufficient evidence to establish a *prima facie* case. Appellants assert that it is mere conjecture on the part of the Office that one of skill in the art would be able to identify the region without undue experimentation. If anything, Dohlsten demonstrates the need to identify the regions in superantigens that are necessary for MHC class II binding, but does not provide reasonable enablement for one of skill in the art to obtain these regions without undue experimentation.

Therefore, in view of the above arguments that Dohlsten is a non-enabling reference and that the Office has not established a *prima facie* case of obviousness, Appellants respectfully request that the Board overturn the rejection of the claims for "non-obviousness".

B. IDS complies with 37 CFR 1.97(c).

The Action contends that the Supplemental Information Disclosure Statement which was submitted on June 21, 2001 did not comply with 37 CFR § 1.97 (c). Appellants traverse.

During the filing of the Notice of Appeal on August 15, 2001, Appellants submitted the Supplemental Information Disclosure Statement with the appropriate fee as indicated in 37 CFR § 1.17(p). Thus, Appellants respectfully request that the Board consider the references contained in the Supplemental Information Disclosure Statement.

IX. CONCLUSION

Appellants have provided arguments that overcome the pending rejections. Appellants respectfully submit that the Action's conclusions that the claims should be rejected are unwarranted. It is therefore requested that the Board overturn the Action's rejections.

Appellants are filing this amended Brief in accordance with 37 CFR § 1.192(d). Appellants assert that the amended Brief satisfies 37 CFR § 1.192(c). Appellants do not believe that there are any additional fees for the filing of this amended Appeal Brief. If however, Appellants are not correct, then the Appellants hereby authorize the Commissioner to deduct any fees for filing of the amended appeal brief from the Fulbright & Jaworski Deposit Account No. 06-2375, Order No. 09804877.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Dated: January 29, 2002

Respectfully submitted,
By _____
Melissa W. Acosta
Registration No.: 45,872
FULBRIGHT & JAWORSKI L.L.P.
1301 McKinney, Suite 5100
Houston, Texas 77010-3095
(713) 651-5407
(713) 651-5246

APPENDIX A

Claims Involved in the Appeal of Application Serial No. 08/765,695

36. A method for the treatment of a diseased condition in a mammal, which condition means the presence of specific cells that are associated with the condition by the expression of a disease specific cell surface structure, wherein one administers to the mammal a therapeutically effective amount of covalent conjugate that is able to activate T lymphocytes to lyse cells that carry the disease specific cell surface structure and comprises:

    a. a biospecific affinity counterpart that is capable of binding to said surface structure, and

    b. a peptide that

        i. contains an amino acid sequence that is derived from a superantigen selected from the group consisting of staphylococcal enterotoxin A, B, C_1, C_2, D and E,

        ii. has the ability to bind to a Vβ of a T cell receptor, and

        iii. has been mutated to show a modified ability to bind to MHC class II antigens compared to the superantigens from which the peptide is derived.

37. The method of claim 36, wherein the disease is selected from the group consisting of cancers, viral infections, autoimmune diseases and parasitic infestations.

38. The method of claim 37, wherein the disease is a cancer.

44. The method of claim 36, wherein the biospecific affinity counterpart comprises polypeptide structure.

45. The method of claim 44, wherein the biospecific affinity counterpart is selected from the group consisting of an antibody or an antigen-binding fragment thereof.

46. The method of claim 44, wherein the biospecific counterpart and the peptide are fused together.

47. The method of claim 45, wherein the biospecific counterpart and the peptide are fused together.

52. The method of claim 36, wherein the superantigen is staphylococcal enterotoxin A.

APPENDIX B

VERSION *

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A01N 37/18, A61K 37/00, 39/02
C07K 3/00, 13/00, 15/00
C07K 17/00, C12N 5/00, 15/00
C12P 21/04, 21/06, C12Q 1/00

A1

(11) International Publication Number: WO 93/14634

(43) International Publication Date: 5 August 1993 (05.08.93)

(21) International Application Number: PCT/US93/00839

(22) International Filing Date: 28 January 1993 (28.01.93)

(30) Priority data:
07/827,540 28 January 1992 (28.01.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/827,540 (CIP)
Filed on 28 January 1992 (28.01.92)

(71) Applicant *(for all designated States except US)*: NATIONAL JEWISH CENTER FOR IMMUNOLOGY AND RESPIRATORY MEDICINE [US/US]; 1400 Jackson Street, Denver, CO 80206 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : KAPPLER, John, W. [US/US]; MARRACK, Philippa [US/US]; 4350 Montview Boulevard, Denver, CO 80207 (US).

(74) Agents: GREGG-EMERY, Valeta, A. et al.; Beaton & Swanson, 4582 S. Ulster Street Parkway, Suite 403, Denver, CO 80237 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: PROTECTIVE EFFECTS OF MUTATED SUPERANTIGENS

(57) Abstract

The present invention includes a method for preventing or treating the toxic effects of a superantigen. A subject is treated with a molecule which interacts with specific Vβ elements of T cells in a manner similar to that of a native superantigen. The molecules of the present invention are mutated or modified superantigens which elicit antibody production without inducing T cell proliferation.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

PROTECTIVE EFFECTS OF MUTATED SUPERANTIGENS

FIELD OF THE INVENTION

This invention relates to methods for preventing and treating antigen-mediated and antigen-initiated diseases. Specifically, it relates to providing protection against superantigen pathogens by administration of molecules which are modified or mutated superantigens which elicit a antibody response against the superantigen without having the pathological effect of the superantigen. The molecules of this invention may also interact with the Vβ elements of T cell receptors in a way which leads to modifications in the way T cells respond to an antigen.

BACKGROUND OF THE INVENTION

The vertebrate immune system evolved to protect vertebrates from infection by microorganisms and large parasites. The immune system responds to antigens in one of two ways: (1) humoral antibody responses, mediated through B cells, involving the production of protein antibodies which circulate in the bloodstream and bind specifically to the foreign antigen which induced them. The binding of the antibody to the antigen makes it easier for phagocytic cells to ingest the antigen and often activates a system of blood proteins, collectively called complement, that helps destroy the antigen; and (2) cell-mediated immune responses, mediated through T cells, involving the production of specialized cells that react mainly with foreign antigens on the surface of host cells, either killing the host cell if the antigen is an infecting virus or inducing other host cells, such as macrophages, to destroy the antigen (Molecular Biology of the Cell (1983), B. Alberts et al. (eds), chapter 17, pp. 952).

The production of antibodies requires a number of preceding events to occur which lead to stimulation of

B cells producing the antibodies. One of the key events involved in the processes leading to antibody production is that of antigen recognition. Antigen recognition requires the participation of thymus (T) cells.

T cells have antigen-specific receptors on their surfaces, termed T cell antigen receptors (TCR). Before T cells can recognize protein antigens, the antigens must be presented on the surface of antigen-presenting cells. The antigens must first be processed by macrophages or other antigen presenting cells. These cells essentially swallow antigens and chop them into peptides which are displayed at the cell surface in combination with major histocompability complex (MHC) molecules.

The major histocompatibility antigens are a family of antigens encoded by a complex of genes called the major histocompatibility complex. MHC antigens are expressed on the cells of all higher vertebrates. In man they are called HLA antigens (human-leucocyte-associated antigens) because they were first demonstrated on leucocytes. There are two principal classes of MHC molecules, class I and class II, each comprising a set of cell-surface glycoproteins. The two classes of MHC antigens stimulate different subpopulations of T cells. MHC class II molecules are involved in most responses to pathogens. In contrast, MHC class I molecules are involved when the pathogen is a virus or when a malignant cell is involved. When MHC class I is involved, antibody stimulation does not result; rather, the interaction of MHC class I processed antigen and T-cell leads to lysis of cells infected with the pathogen.

The processed antigen peptide fits in a cleft on an MHC molecule. Once an antigen is displayed, the few T cells in the body that bear receptors for that particular peptide bind that complex. Most T cells

recognize antigens on the surface of cells only in association with self-MHC glycoproteins expressed on the same cell surface.

The ability of the T cell to complex with the processed antigen and MHC complex is dependent on the T cell receptor (TCR). The TCR consists of two protein chains, α and β. Each chain contains a constant and a variable domain. The variable domains are encoded in two (α) or three (β) different gene segments (variable (V), diversity (D), joining (J)) (Siu et al. (1984) Cell 37:393; Yanagi et al. (1985) Proc. Natl. Acad. Sci. USA 82:3430). In each T cell, the combination of V, D, and J domains of both the α and β chains participates in antigen recognition in a manner which is uniquely characteristic of that T cell and defines a unique binding site. See, Marrack et al. (1988) Immunol. Today 9:308; Toyonaga et al. (1987) Ann. Rev. Immunol. 5:585; Davis (1985) Ann. Rev. Immunol. 4:529; Hendrick et al. (1982) Cell 30:141; Babbitt et al. (1985) Nature 317:359; Buus et al. (1987) Science 235:1353; Townsend et al. (1986) Cell 44:959; Bjorkman et al. (1987) Nature 329:506). Generally, both the α and β chains are involved in recognition of the ligand formed by processed antigen and MHC.

When T cells are stimulated by an antigen, they divide and differentiate into activated effector cells that are responsible for various cell-mediated immune reactions. At least three different reactions are carried out by T cells: (1) cytotoxic T cells specifically kill foreign or virus-infected vertebrate cells; (2) helper T cells help B lymphocytes; and (3) suppressor T cells supress the responses of specific cells.

Recently, it has been shown that a novel class of antigens, termed "superantigens", are able to directly stimulate T cells by binding to a particular Vβ element, that is, the variable domain of the β chain of

the TCR (Kappler et al. (1987) Cell 49:263; Kappler et al. (1987) Cell 49:273; MacDonald et al. (1988) Nature 332:40; Pullen et al. (1988) Nature 335:796; Kappler et al. (1988) Nature 332:35; Abe et al. (1988) J. Immunol. 140:4132; White et al. (1989) Cell 56:27; Janeway et al. (1989) Immunol. Rev. 107:61; Berkoff et al. (1988) J. Immunol. 139:3189; Kappler et al. (1989) Science 244:811). Unlike recognition of conventional peptide antigens, the other components of the T cell receptor (i.e., Dβ, Jβ, Vα, Jα) appear to play little role in the superantigen binding. Superantigens, while generally stimulatory to T cells, appear to interact specifically with particular Vβ elements present on the stimulated T cell. Since the relative number of Vβ genes is limited, many T cells within an individual will bear a particular Vβ element, and a given superantigen is therefore capable of interacting with a large fraction of the T cell repertoire. Thus, depending on the frequency of the responding Vβ population(s), 5-30% of the entire T cell repertoire could be stimulated by a superantigen, whereas the responding frequency to a conventional antigen is usually much less than 1 in 1,000. Although superantigens interact with class II MHC molecules, they appear to act as intact proteins rather than as peptides, that is, they do not appear to bind within the conventional peptide binding groove. Instead, they seem to interact with amino acid residues that are on the outer walls of the binding cleft. Known superantigens and references to their sequences and structures are listed in Table I.

Two distinct classes of superantigen have been described. The first was noted nearly 20 years ago, when Festenstein showed marked responses in mixed lymphocyte reactions between certain MHC identical strains. The stimulating antigens were called minor lymphocyte stimulating (Mls) antigens (Festenstein

(1973) Transplant Rev. 15:62) to differentiate them from MHC antigens. These superantigens are encoded by endogenous retroviral genes (Palmer (1991) Curr. Bio. 1:74). The presence of these genes in the mouse leads to a marked deletion of responding T cells, creating potentially large holes in the animal's T cell receptor repertoire (Pullen et al. (1988) supra). The second set of superantigen is represented by a growing list of bacterial and viral proteins, capable of producing a variety of pathological effects after injection (Marrack & Kappler (1990) Science 248:705).

Staphylococcus aureus (S. aureus), a common human pathogen, produces several enterotoxins, designated as SEA (staphylococcal enterotoxin A) through SEE, which can be responsible for food poisoning and occasionally shock in humans (Marrack & Kappler (1990) supra; Bohach et al. (1990) Crit. Rev. Microbio. 117:251). Some S. aureus isolates also produce toxic shock syndrome toxin-1 (TSST-1), which has been implicated in the majority of cases of human toxic shock syndrome as well as the related exfoliative toxins (ExTF), which are associated with the scalded skin syndrome. Streptococcus pyrogenes, or group A streptococcus, another common human pathogen of the skin and pharynx, also produces toxins with superantigenic properties (Abe et al. (1991) J. Immun. 146:3747). These have been designated streptococcal erythrogenic toxins A-C (SPEA-C).

The amino acid sequence of the S. aureus toxins exhibit some homology, but also exhibit marked differences (See, Bentley et al. (1988) J. Bacteriol. 170:34; Jones et al. (1986) J. Bacteriol. 166:29; Lee et al. (1988) J. Bacteriol. 170:2954; Blomster-Hautamaa et al. (1986) J. Biol. Chem. 261:15783). S. aureus has the ability to stimulate powerful T cell proliferation responses in the presence of mouse cells bearing MHC class II type molecules (Carlson et al.

(1988) J. Immunol. 140:2848; White et al. (1989) Cell 56:27). The S. aureus proteins selectively stimulate murine cells bearing particular Vβ elements.

The binding of toxins to class II MHC molecules is a prerequisite for T cell recognition, but the process is much more permissive for superantigens than that seen with conventional antigens. While peptide antigens are very dependent on allelic MHC residues for binding, the superantigens bind to a wide variety of allelic and isotypic forms of MHC class II molecules (See, Hermann et al. (1989) Eur. J. Immunol. 19:2171; Herman et al. (1990) J. Exp. Med. 172:709; Scholl et al. (1990) J. Immunol. 144:226; Molleck et al. (1991) J. Immunol. 146:463). While T cells rarely recognize peptide antigens bound to self-MHC (allo-MHC) molecules, individual T cell clones can respond to toxins bound not only to various allelic forms of MHC, but also to different class II isotypes and even to xenogenic class II molecules. Such observations suggest that superantigens bind at a relatively conserved site outside the allelically hypervariable groove thought to bind conventional peptide antigens.

Superantigens may contribute to autoimmune diseases, in which components of the immune system attack normal tissue. The process of deletion of T cells responsive to self, potentially harmful self-reactive T cells, is called tolerance or negative selection (Kappler et al. (1987) Cell 49:273; Kapper et al. (1988) Nature 332:35; MacDonald et al. (1988) Nature 332:40; Finkel et al. (1989) Cell 58:1047). The immune system normally deletes self-reactive T cells, but occasionally a few appear to escape the surveillance mechanism. It has been suggested that the ability of superantigens to arouse 20 percent of a person's T cell repertoire could lead to undesirable replication of the few circulating T cells that recognize self (Johnson et al. (1992) Scientific

American 266:92). T cells bearing certain Vβ types have been implicated in various autoimmune conditions, including arthritis and multiple sclerosis. These findings imply that the destructive cells might be activated by a superantigen that binds to the identified Vβ types (Johnson et al. (1992) supra).

Autoimmune diseases are a result of a failure of the immune system to avoid recognition of self. The attack by the immune system of host cells can result in a large number of disorders, including such neural diseases as multiple sclerosis and myasthenia gravis, diseases of the joints, such as rheumatoid arthritis, attacks on nucleic acids, as observed with systemic lupus erythematosus, and such other diseases associated with various organs, as psoriasis, juvenile onset diabetes, Sjögren's disease, and thyroid disease. These diseases can have a variety of symptoms, which can vary from minor and irritating to life-threatening. For example, rheumatoid arthritis (RA) is a chronic, recurrent inflammatory disease primarily involving joints, and affects 1-3% of North Americans, with a female to male ration of 3:1. Severe RA patients tend to exhibit extra-articular manisfestations including vasculitis, muscle atrophy, subcutaneous nodules, lymphadenopathy, splenomegaly, and leukopenia. It is estimated that about 15% of RA patients become completely incapacitated.

Several lines of evidence suggest that T cells specific for self-antigens may play a critical role in the initiation of autoimmune diseases. In the case of RA, the linkage of the disease to the DR4 and DR1 alleles of the class II genes of MHC and the findings that sometimes oligoclonal, activated $CD4^+$ T cells in synovial fluid and tissue of affected joints (Stastny et al. (1976) Engl. J. Med. 298:869; Gibofsky et al. (1978) J. Exp. Med. 148:1728; McMichael et al. (1977) Arth. Rheum. 20:1037; Schiff et al. (1982) Ann. Rheum.

Dis. 41:403; Duquestoy et al. (1984) Hum. Immunol. 10:165; Legrand et al. (1984) Am. J. Hum. Genet. 36:690; Gregerse et al. (1987) Arth. Rheum. 32:15; Burmester et al. (1981) Arth. Rheum. 24:1370; Fox et al. (1982) J. Immunol. 128:351; Hemler et al. (1986) J. Clin. Invest. 78:696; Stamenkoic et al. (1988) Proc. Natl. Acad. Sci. USA 85:1179) suggest the involvement of $CD4^+$, $\alpha\beta$TCR-bearing, class II-restricted T cells in the disease. This view is supported by the finding that partial elimination or inhibition of T cells by a variety of techniques can lead to an amelioration of disease in certain patients (Paulus et al. (1977) Arth. Rheum. 20:1249; Karsh et al. (1979) Arth. Rheum. 22:1055; Kotzin et al. (1989) N. Eng. J. Med. 305:976; Herzog et al. (1987) Lancet ii:1461; Yocum et al. (1989) Ann. Int. Med. 109:863).

U. S. patent application Serial No. 07/732,114, herein specifically incorporated by reference, establishes that specific Vβ elements may be used to diagnose for an autoimmune disease, specifically the presence of a higher percentage of $V\beta14^+$ T cells in synovial fluid may be used to diagnose RA.

Many investigative efforts have focused on developing methods for the treatment of autoimmune diseases. For example, European Patent Publication 340 109, entitled Anti-T-cell receptor determinants as autoimuune disease treatment, and U.S. Patent No. 4,550,086, issued October 29, 1985 to Reinherz et al., entitled Monoclonal antibodies that recognize human T cells, describe a method of detecting a particular sequence of the variable region gene of T cell receptors associated with a particular disease and treating the disease with antibodies to that sequence. U.S. Patent No. 4,886,743, issued December 12, 1989 to Hood et al., entitled: Diagnostic reagents based on unqiue sequences within the variable region of the T cell receptor and uses thereof, describes a method of

diagnosis diseases based on the presence of T cells with a unique sequence in the Vβ region associated with a specific disease. PCT Patent Application Publication WO 90/06758 describes a method for detecting specific Vβ regions associated with RA, specifically, Vβ3, Vβ9, and Vβ10, and for the treatment of RA with monoclonal antibodies which recognize Vβ3, Vβ9, and Vβ10.

Immunity

An animal that has never been exposed to a pathogen has no specific defenses against it. However, the animal can be immunized against the pathogen by injecting it with a non-virulent form of the pathogen with a similar chemical structure as the pathogen but without the ability to cause the pathological effect. The animal will produce antibodies specific against the non-virulent form of the pathogen, and these antibodies can protect the animal against attack from the virulent pathogen.

BRIEF SUMMARY OF THE INVENTION

The present invention includes a method for preventing the toxic effects of a superantigen by treatment with a molecule, wherein said molecule elicits antibody production without inducing T cell activation.

The present invention also includes molecules consisting of mutated or modified derivatives of superantigens.

The present invention further includes a method of modifying T cell response elicited by an antigen comprising administering a molecule which interacts with either the Vβ element alone or both the α and β chains of T cell receptors (TCR).

The molecules of this invention can function by leading to deletion or inactivation/desensitization of

at least one or more subpopulations of T cells which present a particular Vβ element.

To prevent the in vivo toxic effect of superantigen requires an exact understanding of how their effect is achieved. Prior to this invention, while it was known how superantigen interact with T cells, the manner in which a subject animal developed a pathological condition and whether a pathological condition would develop was not well understood. Various observations suggested that any of a number of mechanisms could be the cause of the toxicity.

It has now been found that the pathological condition mediated or initiated by a superantigen can be prevented or treated by administration of the mutant superantigen molecules of the present invention. Administration of the mutant toxins of the present invention may cause antibody production against the mutant molecule. Some of these antibodies also react with the normal non-mutated toxin. Therefore, when the immunized individual is confronted with the normal toxin, these cross-reactive antibodies react with the normal toxin and inhibit its toxic activity.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a schematic ribbon drawing of the three dimensional structure of SEB. Region 1 (residues 9-23), region 2 (residues 40-53), and region 3 (residues 60-61) are differentiated by shading. Sites identified as involved in MHC or TCR binding are shown. Residues identified by mutational analysis as important to MHC and/or TCR binding are indicated.

FIGURE 2a shows the SDS-PAGE analysis of 2 ug of recombinant SEB purified from E. coli and wild-type SEB purified from S. aureus cultures. Molecular mass markers (in kD): β-phosphorylase, 94; bovine albumin,

69; ovalbumin, 45; carboxylase, 30; soybean trypsin inhibitor, 21; lysozyme, 14. FIGURE 2b shows the SDS-PAGE analysis of SEB binding to DR on LG2 cells. Molecular mass markers (in kD): bovine albumin, 69; ovalbumin, 45; chymotrypsinogen, 27; soybean trypsin inhibitor, 21; myoglobin, 17; ~~lysozyme, 14~~.

FIGURE 3 shows the sequences of the SEB mutants. Dashed lines indicate identity to unmutated SEB. The positions of the oligonucleotides used to generate the SEB mutants are also shown.

FIGURE 4 shows the binding of SEB and SEB mutants to DR1-bearing lymphoblastoid line LG2 cells.

FIGURE 5 shows stimulation of T cell hybridomas by region 1 SEB mutants. Preparations of purified SEB or the region 1 mutants were tested for their ability to stimulate a collection of T cell hybridomas bearing of the the Vβ elements known to recognize SEB: KS-20.15 (Vβ7), KS-6.1 (Vβ8.2), KS-47.1 (Vβ8.3), K16-57 (Vβ8.1).

FIGURE 6 shows stimulation of T cell hybridomas by region 2 SEB mutants. Preparations of purified SEB were tested as in Figure 5.

FIGURE 7 shows stimulation of T cell hybridomas by region 3 SEB mutants. Preparations of purified SEB were tested as in Figure 5.

FIGURE 8 shows the effects of SEB and its mutants in vivo. Groups of three mice were weighed and then given balanced salt solution (BSS) containing nothing, 50 ug (left), or 100 ug (right) of recombinant SEB or the mutant SEBs BR-257 or BR-358.

FIGURE 9 shows the protective effect of mutant toxins against challenge with SEB. Mice received doses of either saline or BR-257 three months prior to challenge with wild-type SEB.

DETAILED DESCRIPTION OF THE INVENTION

The molecules of the present invention may be effective in different ways in preventing or treating antigen-mediated or initiated diseases. Some of the different ways in which the molecules of the present invention may be effective include modification of the T cell response and production of antibodies which provide protection against pathogens. Specifically, this invention presents a method of preventing or treating superantigen-mediated or superantigen initiated diseases. The method of this invention generally involves preparing mutated superantigen molecules by methods known in the art and described herein, identifying antigen mutants able to bind either MHC or TCR, and testing for ability to protect against exposure to the non-mutated superantigen.

The present invention describes the feasibility of the above-outlined approach in achieving protection against a known superantigen. Mutants of recombinant Staphylococcal enterotoxin B (SEB) were prepared and purified as described in Examples 1-4 below. SEB mutants able to bind MHC molecules or TCR were selected by examining the binding of mutant SEB molecules to HLA-DR1 homozygous lymphoblastoid line LG2 cells and stimulation of T cell hybridomas bearing different Vβ elements. The SEB mutant BR-257, which bound LG2 cells in a manner indistinguishable from that of non-mutated SEB and did not stimulate T cell hybridomas, injected into experimental animals 3 months prior to exposure to SEB provided complete protection against the toxic effects of SEB. Similar results were obtained in primates.

Although the present disclosure describes production of mutated SEB molecules able to protect animals against subsequent challenge with SEB, the methods of the present invention are equally applicable to other superantigens.

The ability to use the occurrence of specific Vβ elements to diagnose autoimmune diseases, as discussed in detail above, may be combined with the present invention as a method of detecting and treating autoimmune diseases mediated by superantigens. The existence of a superantigen-mediated disease may be determined by a "footprint" analysis, e.g., by determining if there is an alteration in Vβ elements in a disease state. The finding of alterations in Vβ elements, such as the increase in Vβ14$^+$ T cells in synovial fluid in RA, suggests the presence of a superantigen-mediated disease. Techniques known to the art may then be applied in order to isolate and identify the implicated superantigen. The Vβ footprint may be compared against that of a known superantigen for possible implication of that superantigen in initiation or proliferation of the disease. There may a search for genes coding for a superantigen when a virus or bacterial infection is associated with the initiation of the disease. Once a superantigen is identified or isolated, the method of the present invention may be applied to produce a mutant superantigen molecule capable of conferring protection against exposure to the superantigen.

Various terms are used in this specification, for which it may be helpful to have definitions. These are provided herein, and should be borne in mind when these terms are used in the following examples.

As described above, the key event in an immune response is the interaction of MHC molecules with antigens to form a complex presented to T cells. Generally, the T cell response is quite specific, with

only very limited subpopulations of T cells responding to specific complexes of antigen and MHC molecules. The response generally requires interaction of most or all of the components of the T cell receptor. In certain circumstances, however, the presented antigen need only interact with the Vβ element of the receptor, all other components are essentially irrelevant. This means that the antigen can, and does, react with a much greater array of T cells than is normally the case.

The molecules of this invention may interact with the Vβ elements of T cell receptors in a way which leads to modifications in the way T cells respond to a superantigen. "Modifying T cell responsiveness" means that the mutant molecules are able to change the manner in which the subject's T cells respond when provoked by the administered molecule, or to an antigen administered concurrently, previously, or afterward. For example, it is believed that early in the development of T cells, certain subpopulations interact with presented antigens and are deleted. The molecules of this invention can function in this manner, i.e., by leading to deletion or inactivation/desensitization of at least one or more subpopulations of T cells which present a particular Vβ element.

In a particular embodiment of the present invention, the molecules modify the T cell response without changing the B cell response that would normally occur in the subject under consideration. This type of material is useful, for example, for providing passive immunity to a subject, or serving as a vaccine. When superantigen derivatives are used, these derivatives are no longer superantigenic, as they will not provoke a restricted T cell response, but will still serve as antigens in that they generate a B cell response. The superantigen derivatives of the present invention are able to elicit normal antibody production against the superantigen protein.

The molecules of the present invention may also be seen as competitors for other antigens. If the molecules described herein interact with MHC elements otherwise required for generation of a full scale response to an antigen or superantigen, they may prevent or reduce the extent of that response.

The molecules of the present invention may also be viewed as "enhancers" in some instances, where an individual's T cell responsiveness is impaired or weakened by any of a number of causes. Via administration of the molecules encompassed by the present invention, the T cell populations of the individual can be greatly expanded.

The term "modifying T cell responsiveness" as used herein is always relative to a second element (e.g., an antigen), and always refers in particular to responsiveness of T cells presenting a particular Vβ element as part of their T cell receptors, other components of the receptors being essentially irrelevant.

The molecules of the present invention contain, at least, an amino acid sequence of sufficient size to bind to an MHC molecule. The rest of the molecule may consist of amino acids, or may contain carbohydrate or lipid structures.

"Reducing responsiveness" is construed to also include deleting the portion of T cells expressing a particular Vβ element.

"Superantigen derivative" as used herein refers to a molecule whose structure, at the least, contains an amino acid sequence substantially identical to an amino acid sequence presented by a superantigen or portions of a superantigen required for binding to either the MHC ~~or the~~ T cell.

"Modified" superantigen derivative (or fragment), differs from "mutated" superantigen derivative (or ~~fragment~~). The term "modified superantigen" is defined

to refer to molecules which contain an amino acid sequence identical to an amino acid sequence of superantigen, but contain modifications not found in the superantigen molecule itself. For example, if a superantigen contains amino acids 1-250, a "modified" superantigen derivative may contain a sequence identical to amino acids 50-75, positioned in between stretches of amino acids not found in the native superantigen molecule. Additional modifications may include, for example, differing or absent glycosylation patterns, or glycosylation where there normally is none.

"Mutated" superantigen refer to structures where the actual amino acid sequence of the mutation has been altered relative to the native form of the molecule. For example, if a superantigen contains amino acids 1-250, a mutated superantigen may include amino acids 50-68 and 72-75 which are identical to the corresponding native sequence, but differ in amino acids 69-71. The difference may be one of "substitution" where different amino acids are used, "addition" where more amino acids are included so that the sequence is longer than the native form, or "deletion" where the amino acids are missing.

"Vaccine" refers to a formulation when administered to a subject provokes the same type of response typical of vaccines in general, e.g., active immunological prophylaxis. The vaccine may contain adjuvant, or other materials.

It is known that the class of molecules known as superantigens interact with particular Vβ regions of T cell receptors, leading to massive proliferation of particular T cell subpopulations. This interaction, which assumes prior interaction between an MHC molecule and the superantigen, is almost completely independent of any other region of the T cell receptor.

In connection with the interaction of MHC and peptide, it must be noted that MHC molecules are available in a variety of "phenotypes", and different phenotypes are specific for various presented peptides and antigens. For example, HLA-DR is known to be associated with presentation of SEB. Thus, different MHC phenotypes will be of value for different antigens, but determination of HLA phenotype and correlation to presentation of a particular antigen or antigen family is well within the skill of the artisan in this field.

Thus, this invention involves the modification of the T cell response via administration to a subject of a molecule which interacts with both an MHC molecule and at least one Vβ element on T cell receptors. This interaction may affect the T cell response in any number of ways. Perhaps the most elementary manner of affecting the response is one where a molecule interacts with the MHC molecule, preventing the binding of other molecules to the MHC. If the competing molecule has been modified or does not naturally provoke proliferation of T cells, then there will be a lessening or elimination of the response because molecules such as normal antigens or superantigens cannot form the requisite complex with the MHC to generate a T cell proliferative response.

Another manner of modifying the T cell response is via "desensitizing", "inactivating", or "anergizing" the T cells. This mechanism involves interaction of MHC molecule, antigen, and T cell receptor, with subsequent down regulation or inactivation of the T cells. This mechanism is more common in mature subjects than the deletion phenomenon, which occurs in fetal subjects. The latter phenomenon is one where via interaction of the three units, various subpopulations of T cells are in fact removed from the organism.

The modification of the T cell response can also involve stimulation of T cell subpopulations.

Knowledge of the mechanisms described herein permits the artisan to administer to a subject á material which interacts with the MHC and a particular subpopulation of T cells, where proliferation of the T cell subpopulation results. This approach is particularly desirable in the treatment of conditions where a particular Vβ subpopulation or subpopulations are associated with a pathological condition, such as an autoimmune disease.

It should be understood that an immune response, when fully considered, includes both a B cell and a T cell response. One aspect of the invention involves the use of molecules which modify the T cell response without modifying the B cell response. Such materials are especially useful as vaccines, as discussed below.

The molecules of the invention are preferably, but not exclusively, superantigen derivatives. These derivatives may be modified or mutated, as discussed above. These, or any other molecules used herein, are administered in an amount sufficient to modify the T cell response in the manner described. The amount of material used will vary, depending on the actual material, the response desired, and the subject matter of the treatment.

The molecules may also serve as vaccines. These vaccines confer protective immunity on the subject in that they generate a B cell response without the full T cell response normally associated with the normal form of the molecule. Example 7 shows one manifestation of this effect for SEB. Again, depending upon the parameters within the control of the knowledge of the artisan, including the condition being treated, the Vβ molecule to be regulated, and so forth, the material chosen for the vaccine is up to the artisan. The vaccine may contain other materials which are normally found in vaccine compositions, including adjuvants, carriers, etc.

The mode of administration of the materials described herein may vary as well, including intravenous, intraperitoneal, and intramuscular injections, as well as all of the other standard methods for administering therapeutic agents to a subject.

The invention also discloses how to make particular mutants useful in the foregoing methodologies, including isolated nucleic acid sequences coding for mutants, cell lines transformed by these and the vectors and plasmids used therefor, as well as the isolated mutant molecules, including mutant superantigens.

Other applications of the invention described herein will be apparent to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

Polymerase Chain Reaction (PCR) and standard molecular biological methodologies, described in Example 1, were used in the construction and expression of recombinant SEB. SEB mutants were generated in one of two ways, as described in Example 2. The first way introduced random mutations along the entire length of the SEB gene. A second method introduced random mutations in approximately 60-75 base-defined regions of the SEB gene. Initial identification of potential mutant SEBs tested the lysate from transformants for the presence of functional toxin by stimulation of murine T cell hybridomas bearing different Vβ elements in a human DR-expressing cell line. Lysates negative for T cell hybridoma stimulations were tested for the

presence of SEB with the use of monoclonal antibodies (mAbs) against SEB (Example 3). Transformants producing non-functional SEB were sequenced and the mutation identified. Transformants producing mutant SEBs were grown, mutant SEBs purified as described in Example 4. Analysis of the location and effect of the mutation was performed. Since binding to MHC class II molecules is a prerequisite for toxin recognition by T cells, the ability of mutant SEBs to bind human MHC antigen HLA-DR1 was tested as described in Example 5. Mutant SEBs, such as region 3 mutants, were produced which selectively stimulate some, but not all, of the hybridomas bearing specific Vβ elements stimulated by the non-mutated toxin. Thus, the mutated superantigens of the present invention may be used to selectively stimulate only some of the T cell populations stimulated by the wild-type superantigen.

Three regions were identified in the N-terminus part of SEB that affect MHC and/or TCR binding (Example 4 and Figure 1). Mutations in region 1 (residues 9-23) affected both MHC and TCR binding. The results suggested that 23N was particularly important. When the sequences of the S. aureus enterotoxins are aligned for maximum homology (Marrack & Kapple (1990) supra), this residue is conserved among all of the enterotoxins and toxic shock toxin as well. The mutations in region 2 (residues 41-53) drastically reduced the ability of the toxin to bind to MHC class II with a similar effect on their ability to stimulate T cells. About half of the mutations involved F44. Again, this residue is conserved among all the enterotoxins, indicating that this residue probably plays a critical role in the binding of all of the toxins to MHC. Interestingly, none of the mutations in either region 1 or 2 completely obliterated toxin binding to MHC, and in both cases the T cell-stimulating ability of the mutants could be recovered by adding a large excess of

toxin. Mutations in region 3 (60N, 61Y) did not affect binding of the toxins to MHC, but did affect their interaction with two Vβs, 7 and 8.1. This Vβ-specific effect suggests that these amino acids are important for interaction with some, but perhaps not other, TCR Vβs.

The toxicity of mutant SEBs in animals was tested as described in Example 6. Mice were injected with either balanced salt solution (BSS), recombinant SEB, or region 1 SEB mutants at F44 (BR-358) or at N23 (BR-257). Mice receiving 50 ug of either mutant SEB were indistinguishable from those receiving BSS, while those receiving recombinant SEB died within 5 days.

The ability of mutant SEB to provide immune protection from SEB was tested in vivo (Example 7). Mice receiving 100 ug of mutant SEB BR-257 three months prior to challenge with SEB were fully protected from the toxic effect of SEB, whereas those animals not injected with BR-257 died 4-5 days after challenge with SEB. Similar results were obtained with primates. Mutant SEB BR-358 and BR-257 were either ineffective or much less effective in eliciting an emetic response in monkeys (Example 7).

Example 8 describes the application of the above procedure to the SEA toxin and the production of a SEA mutant which behaves the same as the corresponding SEB mutant.

Example 1. Construction and Expression of Recombinant SEB.

Polymerase Chain Reaction (PCR). PCRs (Saiki et al. (1988) Science 239:487) were performed using AmpliTaq recombinant Taq polymerase and the DNA Thermal Cycler from Perkin Elmer Cetus (Norwalk, CT). 20-30 cycles were performed with 1-min denaturing and annealing steps, and an extension step of 1 min for synthesis < 500 bp and 2 min for those > 500 bp.

Template concentrations were 1-10 nM and oligonucleotides primer concentrations were 1 uM. The concentration of the dNTPs was 200 uM, except when attempting to introduce mutations, where the concentration of one of the dNTPs was reduced to 20 mM.

SEB Construct. The gene for superantigen SEB was overexpressed in E. coli as follows. A linearized plasmid containing the genomic SEB gene (Ranelli et al. (1985) Proc. Natl. Acad. Sci. USA 82:5850) was used as a template in a PCR utilizing oligonucleotide primers that flanked the portion of the gene encoding the mature SEB without the signal peptide. The 5′ primer was (SEQ ID NO:1):

5′-TAGGGAATTCCATGGAGAGTCAACCAGA-3′

This primer contains an EcoRI site which places the SEB gene in-frame with the LacZ gene when cloned into plasmid pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ). This oligonucleotide primer also contains an NcoI site which adds an ATG between the LacZ gene fragment and the beginning of the SEB gene so that the SEB gene could be moved easily to other plasmids carrying its own initiation ATG. The 3′ primer contained a HindIII site after the termination codon of the SEB gene (SEQ ID NO:2):

5′-AGCTAAGCTTCACTTTTTCTTTGTCG-3′

The PCR fragment was digested with EcoRI and HindIII and ligated into EcoRI/HindIII-digested pTZ18R. E. coli XL1-Blue (Stratagen, La Jolla, CA) was transformed with the plasmid, a single transformant picked, and the insert (pSEB2) was sequenced to check that it had no mutations.

Upon induction the pSEB2 construct led to overproduction of mostly cytoplasmic SEB (≈ 10 ug/ml of

broth). However, rather than producing a LacZ/SEB fusion protein, the bacteria produced a protein with the same apparent molecular weight as secreted SEB from S. aureus (Fig. 1a). Either the LacZ portion of the fusion protein was cleaved in vivo from the majority of SEB or the ATG introduced between LacZ and SEB was a more efficient translation initiation site than that of LacZ.

Example 2. Generation of SEB Mutants.

SEB mutants were generated in one of two ways. One way introduced random mutations along the entire length of the SEB gene. To do so, the SEB construct of Example 1 was prepared but PCR was performed with concentrations of either dATP or dTTP reduced 10 fold in order to increase Taq polymerase error rate (Innis et al. (1988) Proc. Natl. Acad. Sci. USA 85:9436). This reduces the product amount 5-10 fold. Products of the two reactions were combined, cloned into pTZ18R as described in Example 1, and individual transformants were screened for mutant SEB as described in Example 3 for BR mutants. Of approximately 400 toxin-producing transformants screened, 10 were identified as functional mutants by their reduced ability to stimulate T cells. Low concentrations of dCTP and dGTP were tried as well, but less reduction in product results and no mutants were detected in screening approximately 200 transformants.

A second PCR method was used for introducing random mutations in approximately 60-75 base-defined regions of the SEB gene. The following oligonucleotide primers (A (SEQ ID NO:3), B (SEQ ID NO:4), and C (SEQ ID NO:5)) positioned as shown in Figure 2, were synthesized with each position containing 1% each of the three incorrect bases:

A-: 5'ATTCCCTAACTTAGTGTCCTTAATAGAATATATTAAGTCAAAGTATAG AAATTGATCTATAGA3'

B-: 5'AGCTAGATCTTTGTTTTTAAATTCGACTCGAACATTATCATAATTCCC GAGCTTA3'

C+: 5'CCGGATCCTAAACCAGATGAGCTCCACAAATCTTCCAATTCACAGGCC TGATGGAAAATATGAAAGTTTGTAT3'

These mutant oligonucleotides served as primers in a PCR reaction with either a vector (A and B) or internal SEB (c) oligonucleotide as the other primer, and the SEB gene as the template. Each molecule of synthesized SEB fragment was predicted to have 2-3 random base mutations in the region corresponding to mutant primer. Mutant fragments were incorporated into the SEB gene, either alone or with another fragment containing the 3'-portion of the gene as mixed template in a PCR reaction to resynthesized a full length SEB2 gene (Ho et al. (1989) Gene 77:51; Pullen et al. (1990) Cell 61:1365). Alternatively, this was accomplished by digestion with appropriate restriction enzymes and ligation into pSEB2 from which the corresponding region had been removed.

DNA Sequencing. Plasmid inserts were sequenced directly by the dideoxynucleotide method of Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463, using Sequenase (U.S. Biochemical Corp., Cleveland, OH) and a modification for double-stranded supercoiled plasmid templates (Weickert and Chambliss (1989) in Editorial Comments, U. S. Biochemical Corp., Cleveland, OH, pg. 5-6.

Example 3. Screening of Transformants for SEB Mutants.

Anti-SEB Monoclonal Antibodies (mAbs). 10 mAbs specific for at least five epitopes of SEB were produced by standard methods from B10.Q(βBR) immunized multiple times with SEB. One of these antibodies, B344.1, was used both for quantitation and immunoaffinity purification of SEB and SEB mutants. B344.1 is an IgG1 that was chosen because initial

characterization showed that it had a high affinity for SEB, bound equally well to all of the SEB functional mutants, could detect and immunoprecipitate SEB bound to MHC class II molecules, and did not block T cell recognition of SEB bound to DR (data not shown).

ELISA for SEB. The amount of SEB in preparations wad determined by ELISA. Microtiter wells were coated overnight with a solution of 6 ug/ml natural SEB (Sigma Chemical Co., St. Louis, MO). The wells were then incubated with 25% FCS and washed throughly. Various concentrations of known and unknown SEB preparations were added to the wells as inhibitor followed by a constant amount of anti-SEB antibody (polyclonal rabbit anti-SEB(Toxin Technology, Madison, WI) in BR experiments and monoclonal anti-SEB, B344, in BA, BB, and BC experiments). After 1 hour, the wells were washed thoroughly, and the bound antibody was detected by standard techniques using alkaline phosphatase coupling either to goat anti-rabbit IgG (Sigma Chemical Co.) or to p-nitrophenyl phosphate as substrate. The OD of the reaction at 405 nm was related to the dose of inhibitor and the concentration of the SEB in the unknown estimated by computer analysis of the data.

Initial Screening of Potentially Mutant SEB. For primary screening, totaly lysates were prepared as described from individual transformants containing a potentially mutant SEB gene. Aliquots of each lysate were tested for the presence of functional toxin by stimulation of murine T cell hybridomas bearing α/β receptors with either Vβ7 or Vβ8.3, using human DR-expressing cell lines as presenting cells. Lysates deficient in stimulating either of these hybridomas were assayed for the presence of SEB protein to rule out mutations affecting the level or the full length of the SEB produced. Plasmids from those producing proteins were sequenced to locate the mutation the sequences of the mutants are shown in Figure 2.

The Taq polymerase error-induced random mutants (BR) were clustered in three regions (1, 2, 4), all in the NH_2-terminal 93 amino acids of the molecule (except an additional conservative mutation in one case, BR-374, in the COOH-terminal half of the molecule). As predicted by their method of generation, all but one of these mutations involved a nucleotide substitution of A to G or T to C, and only one silent mutation was found elsewhere in their sequences (data not shown). Additional mutants were generated in region 1 or 2 with mutant oligonucleotide C or A (BC, BA mutants). Region 3 was originally discovered as a single mutant (BA-62) involving the last amino acid covered by oligonucleotide A. The mutant had a different phenotype than the other BA mutants. Additional mutants were produced in this region with mutant oligonucleotide B (BB mutants). Mutations in region 4 were eliminated from further analysis, because it was felt that interfering with the conserved disulfide forming cysteine at position 93 could have far reaching unpredictable effects. In addition, several mutants were not further characterized either because they involved more than one region (Br-474, BA-72), produced highly degraded toxin (BR-267), or were identical to an already existing mutant (BA-50).

Example 4. Preparation of Recombinant SEB.

For initial screening, individual colonies of transformants picked from agar plates were transferred to wells of 96-well microtiter plates containing 100 ul of 2XYT and carbenicillin. A replicate plate was prepared except that the media contained 1 mM IPTG as well. Both were incubated overnight at 37°C with shaking. 50 ul of glycerol was added to each well of the first plate, which was mixed and then stored at -70°C. To prepare SEB-containing lysates, each well of the second plate received 50 ul of HNM buffer (10 mM

Hepes, pH 7.0, 30 mM NaCl, 5 mM $MgCl_2$) containing 3 mg/ml lysozyme and 300 ug/ml DNAse I. The plate was incubated at 37°C for 15 min, frozen, thawed three times, and centrifuged to pellet debris. The supernatants were transferred to a new plate and tested for the presence of SEB both by ELISA and T cell hybridoma stimulation. This method produced preparations containing 0.3 and 10 ub/ml of SEB.

To produce purified mutant SEB, transformants were recovered from the 96-well plate stored at -70°C. Bacteria from overnight cultures (1 vol) containing IPTG were collected by centrifugation, resuspended in a 1:10 vol of HNM buffer containing 1-2 mg/ml lysozyme and 10 ug/ml DNAse I, and frozen and thawed three times. The suspension was centrifuged at 15,000 g for 20 min to remove bacterial debris, and the supernatant was harvested and filtered (0.2 u). The filtrate was passed through a column containing a 1:50 volume of Sepharose 4B beads coupled with 2-3 mg/ml of a mAb to SEB (B344). The beads were washed thoroughly with PBS and the toxin was eluted with 0.1 M glycine-HCl (pH 2.7) and neutralized with 1 M Na_2CO_3. The SEB was concentrated to 1 mg/ml and its buffer changed to BSS using Centricon10 microconcentrators (Amicon Corp., Denvers, MA). This method yielded 3-10 mg of toxin per liter of bacterial culture. SEB and its mutants produced in this manner were > 95% pure as judged by SDS-PAGE. Region 1 SEB mutants are listed in Table II, region 1 and 2 mutants are listed in Table III, and region 3 mutants are listed in Table IV.

The mutations described all involve a nucleotide substitution of A to G, or T to C, which would be predicted by the methodology used for their generation.

When mutations were generated using mutant oligonucleotides C or A, these mutations were concentrated in amino acids 9-23 (Region 1), or 41-53 (Region 2) of the SEB sequence (Table III).

When the oligonucleotide primer B was used, the mutants listed in Table IV were generated.

Amino acid 93 is cysteine in normal SEB. To that end, mutations in this region were not considered further because of the potential interference with disulfide binding. Thus, mutants BR-30 and BR-311 were eliminated.

Those mutants containing changes in more than one region (NOT more than one mutant), i.e., BR-474 and BA-72, were also eliminated, as was BR-267, because the toxin was highly degraded. BA-50 is a known mutant and was not studied further.

Structural Studies of SEB.

The three-dimensional crystal structure of SEB, perhaps the most widely studied member of the staphylococcal enterotoxins, has been recently reported (Swaminathan et al. (1992) Nature 359:801). A schematic drawing of SEB is shown in Figure 1. The SEB molecule contains two domains. The first is composed of residues 1-120 and the second of residues 127-239. As discussed above, three regions have been identified (Kappler et al. (1992) J. Exp. Med. 175:387) in the N-terminus part of the SEB that affect MHC class II binding and/or T cell activation. In each of the regions the specific amino acids that are responsible were determined. Some of the identified residues affect both MHC class II binding and T cell activation, whereas other affect only the latter. As superantigen-MHC class II binding is a prerequisite for T cell activation, residues affecting MHC class II binding will also influence T cell activation, thus no T cell binding information can be inferred from them. But they do provide information about MHC class II binding sites on the superantigen. On the other hand, those residues that influence T cell activation but not MHC

class II binding are likely to be in the T cell binding site on SEB.

Region 1, defined as the stretch of amino acid residues from 9-23, is bifunctional as it affects both TCR and MHC class II binding. Mutations in this region included residues in positions 10, 14, 17 and 23, as either a single or a double mutation (Table II). Asparagine at residue 23 (N23) is on the α-helix, α2, with the side chain pointed towards the solvent. It is the most important residue, being conserved among all staphylococcal enterotoxins and critical for TCR activation. Only five mutations at position 23 affected MHC class II binding, but all of them affected TCR activity. Mutations at residue 14 and 17 affected both MHC class II binding and TCR activation. Residue S14 is on a very short α-helix (α1) and is exposed to the solvent whereas F17 is located at the other end of α1. The locations of S14, F17, and N23 (Figure 1) on the surface of the toxin are favorable for making critical contacts with MHC class II molecules and/or Vβ. Residue F17 points inwards and lies in a loop sandwiched between two other loops, of residues 174-179 and 203-209. This suggests that its replacement by serine (F17S) may have introduced structural changes which reduce MHC class II binding and cytokine production. The association of S14 (on α1) with MHC class II binding, and N23 (on α2) with TCR activity, reveals the structural basis underlying the bifunctional role of region 1. Although the region consists of a small number of sequential amino acids, there are distributed on separated but adjacent elements of the secondary structure that are engaged in different functions. The proximity of α1 and α2 is consistent with the suggestion (Kappler et al. (1992) supra) that the amino acids in region 1 are situated in the trimolecular complex near the junction of Vβ and MHC class II.

Region 2 is defined as residues 40-53 and was suggested to be important in all staphylococcal enterotoxins in mediating binding to MHC class II. About half of the mutations in this region involved the conserved residue F44 (Table III). Other mutations involved residues 41, 45, 48, 52, and 53. These changes affected MHC class II binding and consequently the TCR activation. Thus, this region is probably specific for MHC class II binding. Residues 48-52 are in β-strand β2. Residue F44 is on a turn connecting β1 and β2 with the side chains exposed to solvent. It is situated favorably for engaging in critical hydrophobic binding contacts with MHC class II.

Region 3 is made up of two residues, 60 and 61, and mutation of either one affects the TCR activation but not MHC class II binding. Residues 60 and 61 are in the loop connecting β2 and β3 and are exposed to solvent (Table IV).

Example 5. Binding of Mutant SEB to HLA-DR.

Since binding to MHC class II is a prerequisite for toxin recognition by T cells, the mutations could have affected either the ability of the toxin to bind to DR molecules or the recognition of this complex by the TCR-α/β. To help distinguish these two possibilities, the HLA-DR1 homozygous lymphoblastoid line LG2 was used (Gatti and Leibold (1979) Tissue Antigens 13:35). ^{125}I-labelled LG2 cells were incubated with or without 50 ug/ml recombinant SEB for 2 hours at 37°C. A cell free lysate was prepared in 1% digitonin and incubated for 4 hours at room temperature with Sepharose beads coupled with 3 mg/ml B344 anti-SEB mAb. The beads were washed thoroughly, and the labeled bound material was analyzed by SDS-PAGE under reducing conditions (Laemmlli (1970) Nature 227:680) and autoradiography. As a control, beads bearing the anti-DR mAb, L243 (Lampson and Levy (1980) J. Immunol.

125:293) were used (1/20 the volume of lysate used with the anti-SEB beads).

SEB binds to DR molecules on LG2 (Figure 2b). Immunoaffinity-purified toxins were prepared and assessed for their ability to bind to LG2 using flow cytometry with the same anti-SEB mAb used to purify the SEB and its mutants. 3 x 10^4 LG2 cells were incubated in 100 ul of tissue culture medium overnight at 37°C with vrious concentrations of SEB or its mutants. The cells were washed thoroughly and incubated for 30 min at 4°C with approximately 1 ug/ml of the anti-SEB mAb, B344.1. The cells were washed again and incubated for 15 min at 4°C with fluoresceinated goat anti-mouse IgG1 (Fisher Scientific Co.). The cells were washed again and analyzed for surface fluorescence of the cells corrected for the fluorescence seen with the secondary reagent alone v.s the amount of toxin added. The results, shown in Figure 4, are presented for mutations in each of regions 1, 2, and 3.

The binding by four of the region 1 mutants to LG2 was indistinguishable from that of unmutated SEB. The other three mutants were reduced in their binding capacity by approximately 100-fold. These results suggest that residues between 14 and 23 within region 1 are important in MHC binding. Five of the seven mutations involved residue 23N. In only one case (BR-291, 23N→S) did this mutation reduce MHC binding. These results suggest residue 23N may be important in both MHC binding and Vβ interaction. Region 2 mutants all bound poorly to LG2, approximately 1,000 times poorer than SEB, indicating that region 2 defines a stretch of amino acids, especially 44F, important in binding of the toxin to class II MHC. Region 3 mutants were essentially unaffected in binding to LG2, strongly suggesting that this two-amino acid region (60N, 61Y) is important in Vβ interaction.

Example 5. Effect of Mutations on T Cells Bearing Different Vβ Elements.

The SEB mutants were originally identified because they stimulated either a Vβ7$^+$ or a Vβ8.3$^+$ T cell hybridoma poorly. To assess the effect of the SEB mutations on T cell recognition in more detail, the purified mutant toxins were retested at various doses on additional T cell hybridomas bearing each of the four murine Vβ elements known to recognize SEB (Vβ7, Vβ8.1-3, (White et al (1989) supra; Callahan et al. (1989) supra; Herman et al. (1991) supra). Varying concentrations of toxins were incubated at 37°C overnight with 3 x 10^4 DR$^+$ cells in 200 ul of tissue culture medium. 5 x 10^4 T cell hybridomas of requisite Vβ specificity were added in 50 ul, and the mixture incubated overnight. Response of T cell hybridomas was measured as IL-2 secreted, following Kappler et al. (1981) J. Exp. Med. 153:1198 and Mosmann (1983) J. Immunol. Meth. 65:55. The results are shown in Figures 5-7.

Among the region I mutants (Figure 5), the five involving 23N (BR-267, BR-291, BC-6, BC-66, BC-88) stimulated all of the hybridomas poorly, despite the fact that four of these bound to DR as well as unmutated SEB did. These results indicate that residue 23N is an important amino acid for Vβ interaction, but because the fifth mutant involving this amino acid, BR-291, bound poorly to MHC, this amino acid may influence MHC binding as well. The other two regions 1 mutants also stimulated poorly. In the case of BR-75, this may have been due primarily to its poor binding to DR, but the effect of the BR-210 mutation was several orders of magnitude greater on T cell stimulation than on binding to DR. Taken together, these results are evidence that during T cell recognition of SEB bound to DR, the amino acids in region 1 are situated in the trimolecular

complex at the junction between Vβ and MHC, so that individual residues may interact with either component.

The mutations in the other regions produced less complicated phenotypes. All of the region 2 mutants were defective in stimulation of all of the T cell hybridomas, regardless of the Vβ element in their receptors (Figure 6). There were small differences, but in general the effect of mutations on stimulation was about the same as that seen on DR binding. These results were consitent with the conclusion that mutations in region 2 primarily affect DR binding.

The two-amino acid region 3 mutants were the most discriminating (Figure 7). Despite the fact that random mutants in a 20-amino acid strech flanking this region were generated, all mutations affecting function were found in these two amino acids. These mutants failed to stimulate the hybridomas bearing Vβ7 and Vβ8.1, but not Vβ8.2 or Vβ8.3. To insure that this property was not peculiar to these particular hybridomas, the toxins were tested with four other T cell hybridomas: one $V\beta7^+$. two $V\beta8.1^+$, and one $V\beta8.3^+$. The results were indistinguishable from those in Figure 7 (data not shown).

Example 6. Requirement for T Cell Interaction for In Vivo Effects of SEB.

The question of how important the superantigen properties of the bacterial toxins are to their in vivo toxic effects is unresolved. Previous experiments by the inventors suggested that the toxicity of SEB in mice was related to its ability to stimulate T cells in a Vβ-specific manner, since the toxic effect of SEB was directly related to the frequency of T cells bearing the relevant Vβ elements (Marrack et al. (1990) J. Exp. Med. 171:455). However, the ability of some of S. aureus toxins to bind to class II on monocytes and stimulate the production of cytokines such as TNF and

IL-1 (Parsonnet (1989) Rev. Infect. Dis. 1:263) opens the possibility that direct monocyte stimulation may be sufficient to account for much of the toxin pathology in some situations.

To test this idea, mice were injected with various concentrations of region I mutant BR-257, which binds very well to class II MHC but does not stimulate T-cells except at extremely high levels. Unmutated SEB and mutant BR-358, which like all of the region 2 mutants binds very poorly to class II MHC, were used as controls. To minimize the effects of LPS, which might contaminate the preparations, C3H/HeJ mice were used, a strain defective in LPS responsiveness. Since rapid weight loss is one of the most obvious immediate toxic effects of SEB in mice (Marrack et al. (1990) supra), the mice were weighed daily after the injection on day 0.

Groups of three mice were weighed and then given balanced salt solution (BSS) containing either nothing, 50 ug, or 100 ug of recombinant SEB, mutant SEB BR-257, or mutant SEB BR-358. The mice were weighed daily at the same time of day until they died. The results are shown in Figure 8. Results are presented as the average percent change from the starting weight for the surviving mice.

Mice given either 50 or 100 ug of recombinant SEB lost weight rapidly over 3-4 days, and all of the mice were dead by day 5. Mice given mutant BR-358 showed no effects and were indistinguishable from those given BSS alone. Mice given 50 ug of BR-257 were unaffected as well; however, those given 100 ug of BR-257 showed a slight weight loss followed by recovery.

These results confirm that in mice the majority of the toxicity of SEB is dependent on its ability to stimulate T cells, suggesting that T cell-derived lymphokines themselves or those produced by other cells activated by T cells are very important in the mode of

action of this toxin. However, the small effect of BR-257 at the higher dose raises the possibility of a contribution from class II-bearing cells directly stimulated by bound SEB without T cell involvement.

Example 7. Protective Effect of SEB Mutants.

The protective effect of SEB mutants was tested. In these experiments, mice received doses ~~of saline~~ solution or 100 ug BR-257 three months prior to a challenge with wild-type SEB. On the day of the challenge (day "0"), the mice received 50 ug of SEB intraperitoneal. Weight change and survival were measured. Results are shown in Figure 9.

All mice which had received the control died 4-5 days after challenge with SEB, whereas there was a protective effect shown in the mice which had been immunized with the SEB mutant.

Example 8. Production of SEA Mutants and Their Protective Effects in Animals.

Staphylococcal enterotoxin A (SEA) mutants were produced according to the procedures described above. Superimposing the amino acid sequence of SEA on that of SEB, it has been bound that a mutation at position 45 inhibits SEA's ability to bind to MHC, in a similar manner to that observed with the position 45 SEB mutant.

Similar studies were conducted with primates. Monkeys received either wild-type SEB, or either of the reion 1 SEB mutants BR-257 (mutated at F44) or BR-358 (mutated at N23), and the induction of an emetic response assessed. Both mutant SEB molecules were either ineffective or much less effective in inducing an emetic response in primates, than wild-type SEB.

These results confirm that the method of producing mutant superantigen described in this disclosure is applicable generally to all superantigens, and provides

a method of protecting patients from the pathological effect of superantigens.

TABLE I. KNOWN SUPERANTIGEN SEQUENCES AND STRUCTURES

Staphylococcus	Staphylococcal enterotoxin A	Huang et al (1987) J. Biol. Chem. 262:7006 Betley et al. (1988) J. Bacteriol. 170:34
	Staphylococcal enterotoxin B	Jones & Khan (1986) J. Bacteriol. 166:29 Huang & Bergdoll (1970) J. Biol. Chem. 245:3518 Ranelli et al. (1985) Proc. Natl. Acad. Sci. 82:5850
	Staphylococcal enterotoxin C1 and C3	Schmidt & Spero (1983) J. Biol. Chem. 258:6300 Bohach & Schlievert (1987) Mol. Gen. Genet. 209:15 Couch & Betley (1989) J. Bacteriol. 171:4507
	Staphylococcal enterotoxin D	Bayles & Iandolo (1989) J. Bacteriol. 171:4799
	Staphylococcal enterotoxin E	Couch et al. (1989) J. Bacteriol. 170:2954
Toxic Shock Toxin		Schlievert et al. (1981) J. Infect. Dis. 143:509 Blomster-Hautamaa et al. (1986) J. Biol. Chem. 261:15783 Bergdoll et al. (1981) Lancet 1:1017
Exfoliating Toxins		Lee et al. (1987) J. Bacteriol. 169:3904
Streptococcus	Streptococcal pyrogenic toxin C	Goshorn & Schlievert (1988) Infect. Immun. 56:2518 Tomai et al. (1990) J. Exp. Med. 172:359
Mouse Mammary Tumor Virus		Fasel et al. (1982) EMBO J. 1:3 Donehower et al. (1981) J. Virol. 37:226 Donehower et al. (1983) J. Virol. 45:941 Raceviskis & Prakash (1984) J. Virol. 51:604 Choi et al. (1991) Nature 350:203 Acha-Orbea et al. (1991) Nature 350:207 Pullen et al. (1992) J. Exp. Med. 175:41 Moore et al. (1987) J. of Virology 61:480

TABLE II. REGION 1 SEB MUTANTS.

Mutant Name	Position	Change(s)
BR-75	F17	Phe-Ser
BR-210	S14	Ser-Leu
BR-257	10, N23	Asp-Asn; Asn-Asp
BR-291	N23	Asn-Ser
BR-358	F44	Phe-Ser
BR-374	D48, 160	Asp-Gly; Leu-Val
BR-30	Y91	Tyr-Cys
BR-311	C93	Cys-Arg
BR-474	46, C93	Tyr-Ser; Cys-Arg
BR-267	F44, 54, 55	Phe-Ser; Lys-Arg; Asp-Val

TABLE III. REGION 1 AND 2 SEB MUTANTS GENERATED WITH MUTANT OLIGONUCLEOTIDES A OR C.

Mutant Name	Position	Change(s)
BC-6	N23	Asn-Ile
BC-66	N23	Asn-Tyr
BC-88	N23	Asn-Lys
BA-3	F44	Phe-Cys
BA-15	L45	Leu-Val
BA-24	41, 53	Ile-Arg; Gln-Val
BA-31	46, 52	Tyr-Leu; Ser-Phe
BA-50	F44	Phe-Ser
BA-53	F44, 43	Phe-Leu; Ile-Arg
BA-62	Y61, 189	Gln-Ser; Ile-Arg
BA-72	L45, N60	Leu-Tyr; Asn-Lys

TABLE IV. REGION 3 SEB MUTANTS

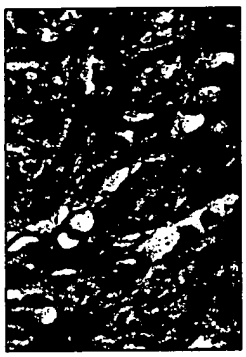
Mutant Name	Position	Change(s)
BB-14	36, Y61	Gln-Leu; Tyr-Cys
BB-21	N60	Gln-Asn
BB-47	Y61	Tyr-Gln

APPENDIX C

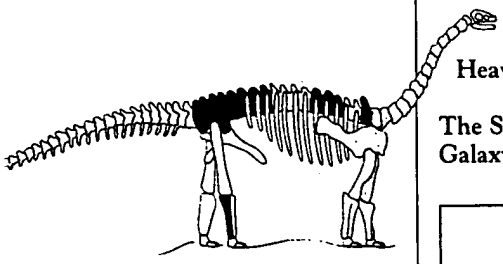
ISSN 0036-8075
16 DECEMBER 1994
VOLUME 266
NUMBER 5192

SCIENCE

AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE



1803 & 1865
Herpes link to Kaposi's?



1805
It could be a contender

NEWS & COMMENT

RESEARCH NEWS

POLICY FORUM

PERSPECTIVE

ARTICLE

RESEARCH ARTICLE

DEPARTMENTS

Toxic Shock Syndrome Toxin–1 Complexed with a Class II Major Histocompatibility Molecule HLA-DR1

Jongsun Kim, Robert G. Urban, Jack L. Strominger, Don C. Wiley*

The three-dimensional structure of a *Staphylococcus aureus* superantigen, toxic shock syndrome toxin–1 (TSST-1), complexed with a human class II major histocompatibility molecule (DR1), was determined by x-ray crystallography. The TSST-1 binding site on DR1 overlaps that of the superantigen *S. aureus* enterotoxin B (SEB), but the two binding modes differ. Whereas SEB binds primarily off one edge of the peptide binding site of DR1, TSST-1 extends over almost one-half of the binding site and contacts both the flanking α helices of the histocompatibility antigen and the bound peptide. This difference suggests that the T cell receptor (TCR) would bind to TSST-1:DR1 very differ ntly than to DR1:peptide or SEB:DR1. It also suggests that TSST-1 binding may be dependent on the peptide, though less so than TCR binding, providing a possible explanation for the inability of TSST-1 to competitively block SEB binding to all DR1 molecules on cells (even though the binding sites of TSST-1 and SEB on DR1 overlap almost completely) and suggesting the possibility that T cell activation by superantigen could be directed by peptide antigen.

Toxic shock syndrome toxin–1 (TSST-1) is a 22-kD protein superantigen secreted by *S. aureus* that causes toxic shock in humans probably by polyclonal activation and lymphokine secretion from T cells (*1*). Patients exhibit selective expansion of T cells (up to 50% of the total) expressing the $V_\beta 2$ family of TCR β chains (*2*). Like other bacterial and viral superantigens, TSST-1 cross-links the class II major histocompatibility complex (MHC) proteins of antigen-presenting cells to the V_β chains of the antigen receptor of T cells (*3*). Earlier studies indicate that superantigens bind primarily outside of the peptide-antigen binding groove of class II MHC molecules and to mainly conserved regions of TCRs, including a fourth region of hypervariability (CDR4) on V_β chains (*2, 4*). Furthermore, bacterial superantigens function as intact molecules, unlike conventional antigens, which are degraded to a short peptide and complexed with an MHC molecule for recognition by T cells (*5*). Their binding mode may circumvent the clonal specificity of T cells by binding away from the six major TCR hypervariable loops, enabling superantigens to activate a large fraction of all T cells (10 to 30%, hence their name) that bear certain families of TCR V_β chains (*6*).

The x-ray crystal structure of a complex between the superantigen *S. aureus* enterotoxin B (SEB) and the human class II MHC molecule HLA-DR1 was determined recently (*7*). It revealed that SEB binds exclusively to the α chain of DR1 off one edge of the peptide binding groove. One loop of SEB covers residues of DR1 recognized by the TCR during conventional antigen recognition (*8*), which suggests an unconventional model for the interaction between the TCR and MHC during superantigen activation (*7*). Although they are only 16% identical in sequence (*9*), TSST-1 and SEB have very similar three-dimensional structures (*9, 10*). However, sequence differences in TSST-1 at residues corresponding to SEB residues involved in binding to DR1 suggest the existence of substantially different superantigen-DR1 interactions (*7*), although mutation and competition studies suggest substantial overlap in the binding site on DR1 (*11–15*).

Here, we determined the three-dimensional structure of the TSST-1:DR1 complex by x-ray crystallography. The locations of the DR1 and TSST-1 molecules in a crystal of the TSST-1:DR1 complex were both determined by two independent methods with the use of coordinates of the individual molecules (*9, 16*). First, their locations were each found by separate, six-dimensional searches of α carbon models (*17*) through a single isomorphous re-

Table 1. Data processing and refinement statistics. HLA-DR1 (*27*) was cocrystalized with TSST-1 in a 1:1 molar ratio (final total protein concentration of ~12 mg/ml, 10 mM tris buffer, pH 7.5). Crystals of lyophilized TSST-1 (Sigma or Toxin Technology) grown by vapor diffusion from 100 mM acetate (pH 5.5), 17% polyethylene glycol (4 kD), and 5% ethylene glycol (or methyl-propanediol) at room temperature have space group $I4_1$ with unit cell dimensions of $a = b = 144.08$ Å and $c = 106.55$ Å. Diffraction data to 3.5 Å resolution from a native crystal and from one heavy atom derivative [soaked in 3 mM ethylmercury-thiosalicylate (EMTS) solution for 3 hours] were collected at room temperature with the use of an MAR-research detector with CuKα x-rays generated by an Elliot GX-13 rotating anode with Franks double mirror optics. Data were processed and scaled with the program XDS (*28*). The heavy atom position was determined from difference Patterson maps and was confirmed by difference Fourier analysis with the molecular replacement phases calculated from either the DR1 molecule alone or the TSST-1 molecule alone with the use of the program ROCKS (*29*). The heavy atom binding site is common to that of other DR1 crystal forms (*7, 16*). Heavy atom parameters were refined and single isomorphous replacement (SIR) phases calculated with the program PHARE. SIR phases were improved by solvent flattening (*30*) (50% solvent) to give a 3.5 Å electron density map in which DR1 and TSST-1 were located by a six-dimensional real space search (*17*). DR1 and TSST-1 locations were revealed as peaks nine and five times, respectively, the standard deviation in independent, six-dimensional search functions. DR1 and TSST-1 positions were also independently determined by molecular replacement methods with the use of the program package X-PLOR (*18*). The correlation coefficients calculated for the highest peaks in the DR1 and TSST-1 rotation functions were 0.11 and 0.10, both twofold greater than the next highest peaks. The translation function values were 0.22 and 0.31, respectively, with standard deviations of 0.012. *R* factors calculated from DR1 or TSST-1 alone were 49.7 and 48.6%, respectively. The relative locations of the DR1 and TSST-1 molecules along the crystallographic *z* axis were determined by inspection of the heavy atom binding sites determined by difference Fourier analysis with the use of either DR1 model phases or TSST-1 model phases. The *R* factor for the TSST-1:DR1 complex thus located was 43%. The location of DR1 and TSST-1 determined by molecular replacement and independently by the six-dimensional search of the SIR map were the same, revealing one TSST-1:DR1 complex per asymmetric unit. A further indication that the location of DR1 was correct was the discovery in this crystal of the same dimer of DR1 molecules seen in three earlier crystals containing DR1 (*7, 16*), this time positioned on a crystallographic twofold symmetry axis. f_H, heavy atom structure factor; *E*, residual lack of closure; FOM, mean figure of merit; rms, root mean square.

Data set	Resolution (Å)	Data coverage (%)	R_{merge}* (%)	R_{iso}† (%)	FOM	rms f_H/E
Native	3.5 (3.7 to 3.5)	92.2 (92.8)	10.8 (27.8)			
Derivative	3.5 (3.7 to 3.5)	95.5 (98.3)	12.9 (32.8)	20.0	0.40	1.51

*$R_{merge} = \Sigma_{hkl}\Sigma_{obs} | I^{hkl}_{obs} - \langle I^{hkl}\rangle | / \Sigma_{hkl}\Sigma_{obs} I^{hkl}_{obs}$ where I_{obs} is the observed intensity. †$R_{iso} = \Sigma_{hkl} | F_{native} - F_{derivative} | / \Sigma_{hkl} F_{native}$.

J. Kim and D. C. Wiley, Howard Hughes Medical Institute and Laboratory of Molecular Medicine, Children's Hospital, Boston, MA 02115, USA, and Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA.
R. G. Urban and J. L. Strominger, Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA.
*To whom correspondence should be addressed.

placement, solvent-flattened 3.5 Å electron density map. The same locations were found by independent rotation and translation function calculations (*18*) for each protein in the TSST-1:DR1 crystal (Table 1).

The current model contains 94% of the residues of DR1, 13 alanine residues representing antigenic peptides bound to DR1, and the entire 194 amino acids of TSST-1. (Residue numbers are prefixed with α and β to indicate the α and β subunits of the DR1 molecule, and p and t to indicate the antigenic peptide bound on DR1 and TSST-1, respectively.) The model is presently refined to a crystallographic *R* factor of 0.22 (10 to 3.5 Å, R_{free} = 35%) (Table 2). Because significant intensity data were unmeasurable beyond 3.5 Å resolution, high-resolution details, such as the certainty of hydrogen bonding or salt bridge formation, which require a high-resolution refined structure, cannot be reliably assessed from the current model. However, a series of simulated annealing omit maps calculated around the contact regions clearly shows that the location of side chains and the composition of the interfaces in the TSST-1:DR1 complex are evident. DR1 crystallizes as a dimer of the αβ heterodimer, as has been observed in other DR1 crystal forms (*7*, *16*).

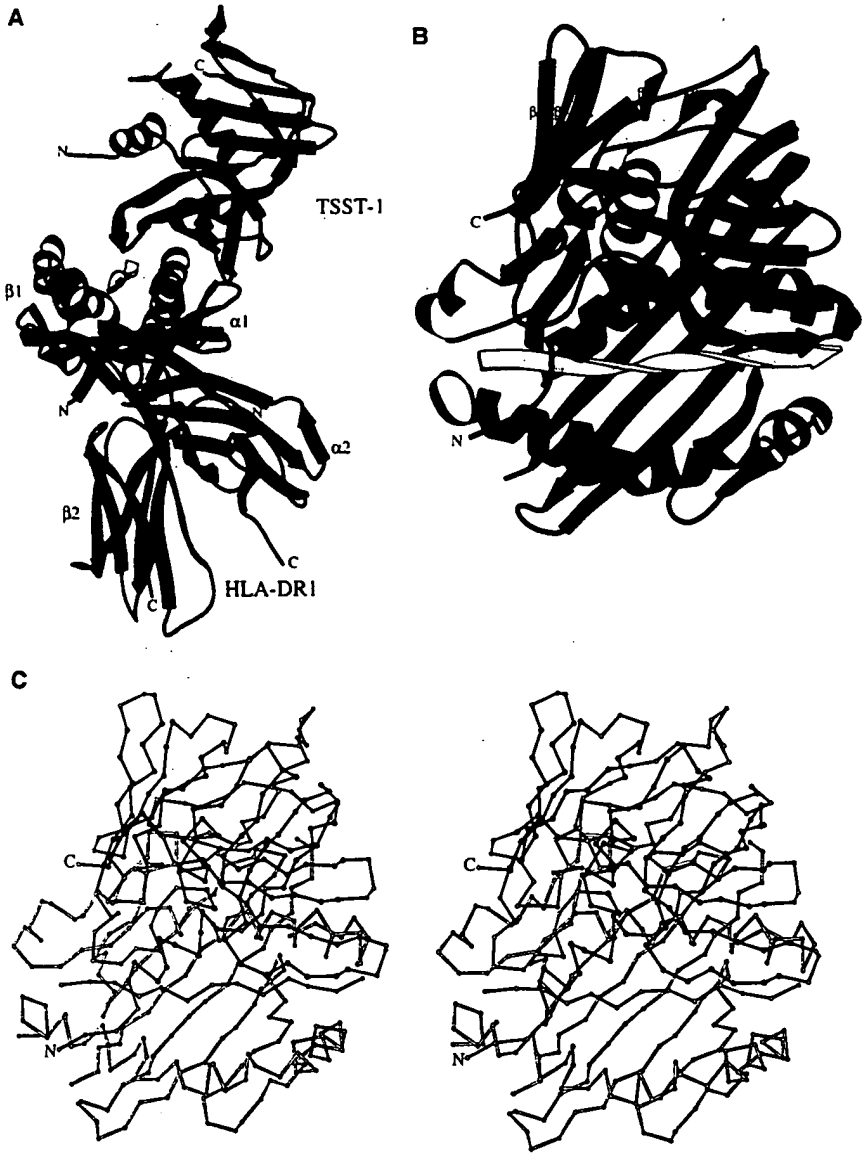


Fig. 1. (**A**) The TSST-1:DR1 complex [all the figures are generated with MolScript (*32*)]. The α and β subunits of DR1 are light blue and blue, respectively, the antigenic peptide is yellow, and TSST-1 is red. (**B**) The TSST-1:DR1 complex viewed toward the MHC peptide binding site of DR1 [coloring is as in (A)]. Secondary structural elements of TSST-1 (α1 and α2 and β1 through β12) are marked for comparison with (C). (**C**) Stereo view of the TSST-1:DR1 complex viewed as in (B). The α1 and β1 domains of DR1 are in white, the antigenic peptide is shaded, and TSST-1 is black. The α2 and β2 domains of DR1 are omitted for clarity. The secondary structural elements of TSST-1 are marked by closed circles for the α carbons: t5 through t14 (α1), t18 through t28 (β1), t32 through t37 (β2), t41 through t47 (β3), t60 through t75 (β4), t79 through t89 (β5), t101 through t106 (β6), t109 through t111 (β7), t119 through t124 (β8), t125 through t140 (α2), t152 through t158 (β9), t162 through t167 (β10), t180 through t182 (β11), and t187 through t193 (β12). C, COOH-terminus; N, NH_2-terminus.

The overall structure of the TSST-1:DR1 complex is shown in Fig. 1. The NH_2-terminal β barrel domain of TSST-1 is primarily involved in complex formation between DR1 and TSST-1 molecules, as had been suggested by x-ray crystallographic analysis of TSST-1 (*9*, *10*) and peptide studies mapping the TSST-1:MHC interaction (*19*). The COOH-terminal domain of TSST-1 is oriented up and away from the DR1 molecule (Fig. 1A). The TSST-1 molecule covers the entire top of the DR1 α1 domain and about half of the peptide binding groove (Fig. 1, B and C).

Although a continuous surface, the TSST-1:DR1 interface (Fig. 2A) can be usefully divided into three major contact regions: an interdigitation of two loops from TSST-1 (near t30 and t50) with two loops from DR1 (near α18 and α38) (contact region I), the packing of four β strands of TSST-1 on the top of the α helix of the α1 domain of DR1 (contact region II), and an interaction between the antigenic peptide bound on DR1 and two β strands from TSST-1 (contact region III). Twenty-four residues of TSST-1 and 20 residues of DR1 (Table 3), making hydrophobic interactions, hydrogen bonds, and salt bridges, form an extensive contact [~1000 $Å^2$ buried with the use of a 1.4 Å solvent probe (*20*)].

Contact region I (Fig. 2B) is centered on Leu^{30} of TSST-1, which is surrounded by nonpolar residues (M36, I63, and Y13) (*21*) of DR1. This leucine is conserved in SEB (L30 of TSST-1 = L45 of SEB) (Table 3) and makes similar contacts to DR1 in the SEB:DR1 interface (*7*). In both the TSST-1:DR1 and SEB:DR1 interfaces, this leucine is one of the most extensively buried residues (159 $Å^2$ in TSST-1; 80 $Å^2$ in SEB). To one side (left, Fig. 2B) of the leucine, polar residues (D27 and K58) of TSST-1 potentially hydrogen bond to polar residues (Q18, Y13, and K67) of DR1. On the other side of the leucine (right, Fig. 2B), K39 of DR1 potentially hydrogen bonds to S53 and the main chain carbonyl oxygen of P50 on TSST-1. In the SEB:DR1 interface, the same DR1 K39 formed a very different contact, forming a salt bridge to SEB E67 (*7*) (Fig. 3). Mutation of DR1 K39 has been shown to disrupt binding of both TSST-1 and SEB (*14*, *15*).

In contact region II, the top face of four turns of the α helix on the DR1 α1 domain (α57 to α71) (Fig. 2C), a site also recognized by T cell receptors, is covered by four

β strands of TSST-1 (β2, β3, β5, and β4) (Fig. 1B). Six of the 10 DR1 residues in the contact (α57, α60, α61, α63, α64, and α67) when mutated affect T cell stimulation by conventional antigens (8). The same six DR1 residues are contacted by SEB, but the contact is made by SEB disulfide loop residues 94 to 97 (7), which are deleted in TSST-1 (replaced by the short loop between β4 and β5) (Fig. 1B). In the TSST-1:DR1 interface, five of the 10 DR1 helical residues are highly conserved in class II MHC sequences and five are polymorphic (22). About 63% of the buried surface in region II is contributed by the conserved DR1 residues, which may be enough to stabilize this contact in TSST-1 complexes with other class II MHC molecules. A cluster of nonpolar residues on the concave surface of the TSST-1 β sheet (I42, L44, I46, P50, I81, and F83) (10) interact with most DR1 α-helical residues; at one end of this interface, the charged DR1 residues E71 and K67 potentially form a salt bridge and hydrogen bond with TSST-1 R34 and the main chain at D27, respectively (Fig. 2C). L46 of TSST-1, which is in the center of the apolar core of contact region II (Fig. 2C), is homologous to E67 of SEB, which in the DR1:SEB complex is connected by a salt bridge to K39 in contact region I of DR1.

In contact region III (Fig. 2D), a β loop of TSST-1 (β4 and β5) makes a few interactions with the COOH-terminal region of the bound peptides and the top of one turn of the β1 domain α helix. Residues 7 to 13 (p7 to p13) of the peptide are near TSST-1 residues K70, Q73, I81, and F83, and residues T75 and S76 appear close enough to hydrogen-bond to p13. Q73 of TSST-1 may hydrogen-bond to DR1 β chain residues Y60 and Q64 (Fig. 2D). Q73 of TSST-1 is homologous to C93 of SEB. In the SEB: DR1 interface, that SEB residue (C93) contacts the top of the α1 domain α helix and thus resides in contact region II rather than in region III, as in TSST-1:DR1.

Although the structures of TSST-1 and SEB are very similar (9, 10), SEB contacts only DR1 at two regions—on one side of DR1 (contact region I) and on top of the α

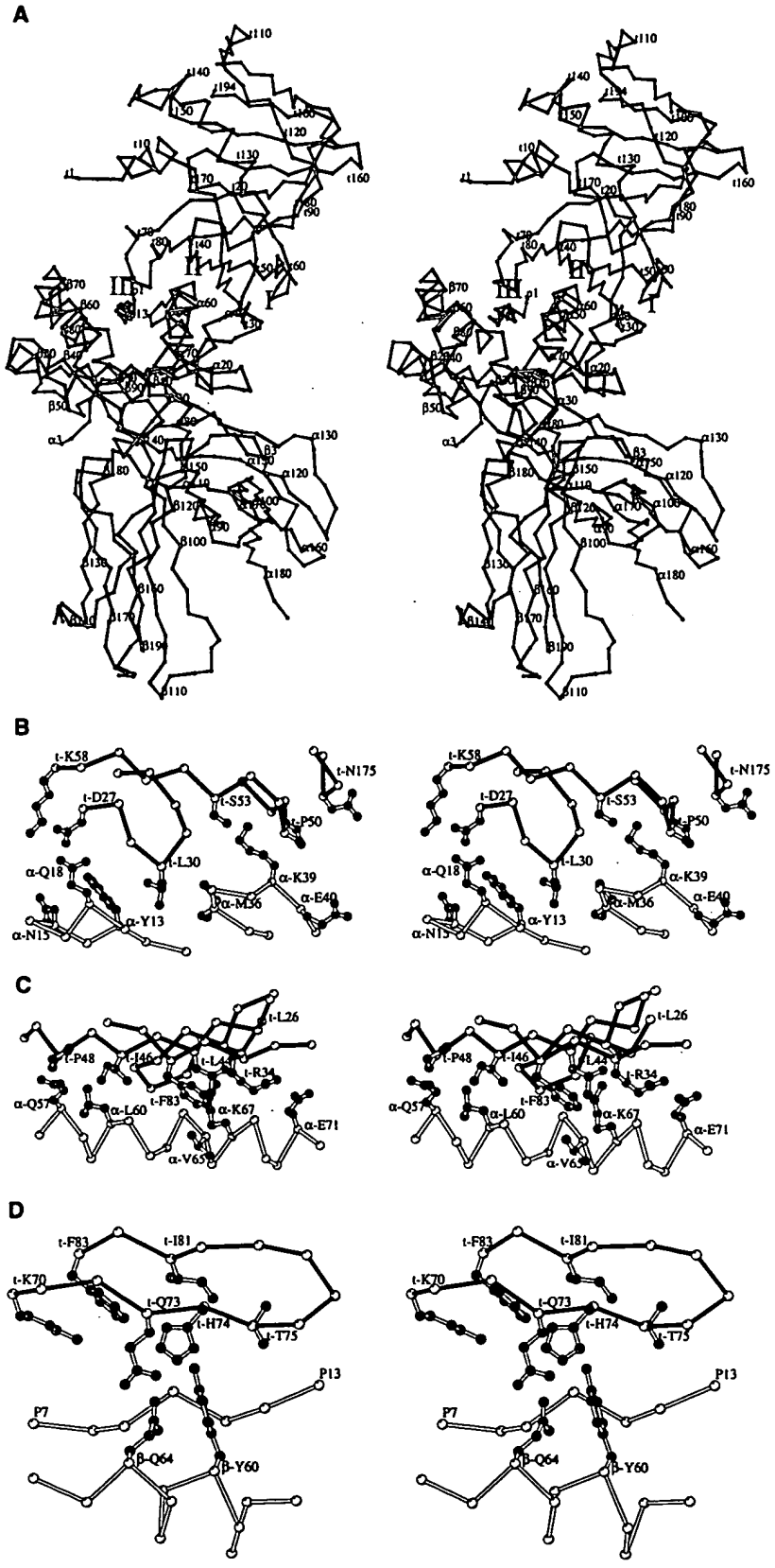


Fig. 2. (**A**) Three major contact regions in TSST-1:DR1 are designated by I, II, and III, respectively (33). The Cα positions of DR1 are indicated by open circles and those of TSST-1 by closed circles. (**B**) Contact region I. The DR1 and TSST-1 Cα chains are indicated by light bonds and dark bonds, respectively. Contact region I is formed by interdigitation of two loops from the DR1 molecule (α13 to α18 and α36 to α39) and two loops from the TSST-1 molecule (t27 to t34 and t42 to t58). Some residues from the COOH-terminal domain of TSST-1 are also located in this region. (**C**) Contact region II. DR1 and TSST-1 Cα chains are indicated by light bonds and dark bonds, respectively. Four β strands from the NH_2-terminal domain of TSST-1 cover the α helix from the α1 domain of DR1. (**D**) Contact region III. DR1 and TSST-1 Cα chains are indicated by light bonds and dark bonds, respectively. TSST-1 interacts with the COOH-terminal region of the antigenic peptide (p7 to p13) and some residues from the β1 domain α helix of DR1.

chain α helix (contact region II)—whereas TSST-1 has one further contact region, to the bound peptide and the DR1 β chain. Both TSST-1 and SEB use homologous leucines (L30 TSST-1 and L45 SEB) to form contact region I, but TSST-1 uses residues (like L46 and F47) that were part of contact region I in SEB:DR1 to form contact region II on top of the DR1 helix, and TSST-1 uses residues (like Q73) that were part of contact region II (top of the α helix) in SEB:DR1 to reach over the top of the peptide and β chain to form contact region III.

Structural models for the interaction of TCRs with superantigens have been proposed on the basis of mutation studies and the three-dimensional structures of SEB, TSST-1, and the SEB:DR1 complex (*7*, *10*). Sites on TSST-1 where mutations affect TCR binding (Fig. 3) form a surface facing up away from the DR1 molecule in the TSST-1:DR1 complex. The very different mode of binding of TSST-1 to class II MHC molecules seen here, relative to SEB, suggests that TCRs may be oriented very differently in complexes with various superantigens (*23*), yet still may be capable of initiating a signal solely on the basis of crosslinking MHC-bearing membranes to TCR-bearing membranes. Although the structures of the complexes of SEB and TSST-1 with DR1 both suggest that TCRs could simultaneously contact superantigen and DR1 molecules, the TSST-1:DR1 complex also suggests the possibility that TCRs might contact only TSST-1 and be blocked from contacting DR1.

The binding of TSST-1 to class II MHC molecules is known to be affected by changes in the α1 and β1 domains of DR molecules (*14*, *24*) and to mutations at residues α36 and α39 (*14*, *25*), which is consistent with the interface observed in the TSST-1:DR1 crystals (Figs. 1 and 2). A comparison of the structure of the TSST-1:DR1 complex with that of the SEB:DR1 complex (*7*)

Table 2. Refinement statistics. TOM/FRODO (*31*) was used for model building, and X-PLOR (*18*) was used in the refinement with PARAM19X.PRO as the parameter file. Four refinement steps were carried out with the available 3.5 Å resolution data. The initial molecular replacement model refined as rigid molecules had a value for R_{cryst} of 43%. Residues in poor electron density in the map calculated from combined SIR and model phases were eliminated from the model. Cycles of positional and individual *B* factor least squares refinement reduced the *R* factor to 25% and R_{free} to 38%. Improvements in phases allowed the omitted residues to be rebuilt from inspection of $2F_o - F_c$ and $F_o - F_c$ electron density maps. After rebuilding, a simulated annealing (3000°) and individual *B* factor refinement reduced the value for R_{cryst} to 22%. Although the value for R_{free} did not drop significantly at this stage, significant improvement of electron density was observed. After rebuilding into the resultant $2F_o - F_c$ map, the initial *R* factor was 38%, and subsequent positional and individual *B* factor refinement reduced the *R* factor to 22% and R_{free} to 35%. As a precaution against model bias in placing side chains, a series of simulated annealing omit maps (*18*) was examined. The absence of intensity data beyond 3.5 Å resolution limits the effectiveness of this refinement, so that the precision of atomic positions and the certainty of side chain placement is reduced. R_{free} was calculated with ~10% of reflections excluded from the refinement. The number of reflections in the refinement was 10,546 (with $F_{obs} > 0$); the number of reflections in the free set was 1050; the number of atoms in the refinement was 4654; and the number of solvent molecules was 0.

Structural statistics	Before refinement	After partial refinement
R_{cryst}* (10.0 to 3.5 Å)	0.38	0.22
R_{free}* (10.0 to 3.5 Å)	0.38	0.35
Root mean square deviation		
Bond lengths (Å)	0.02	0.018
Bond angles (degrees)	2.7	3.5
Dihedral angles (degrees)	31.0	27.0
Improper torsion (degrees)	1.6	1.5

*R_{cryst} and $R_{free} = \Sigma_{hkl} \| F_{obs} | - | F_{calc} \| / \Sigma_{hkl} | F_{obs} |$, where F_{obs} is the observed structure factor.

Table 3. The TSST-1:DR1 interface. Underlined residue numbers are also found in the DR1:SEB interface.

TSST-1 residue (SEB residue no.) [buried surface area (Å²)]	Location	DR1 contact residues* [buried surface area (Å²)]
t-D27† (D42) (35.8)	β1	α-Q18 (79.4); α-Y13† (13.8); α-K67† (93.5)
t-S29 (F44) (29.8)	β1β2 loop	
t-L30 (L45) (156.9)	β1β2 loop	α-M36 (45.0); α-I63 (19.7)
t-G31 (Y46) (34.2)	β1β2 loop	
t-S32 (F47) (31.0)	β2	α-A64 (54.2)
t-R34‡ (L49) (93.9)	β2	α-E71‡ (51.5); α-A68 (41.3)
t-I42 (N63) (11.5)	β3	
t-L44 (R65) (49.0)	β3	
t-I46 (E67) (74.3)	β3	α-A61 (26.7); α-Q57 (85.1)
t-F47† (F68) (15.2)	β3	α-K39† (120.4)
t-P48 (K69) (60.9)	β3β4 loop	α-L60 (53.1)
t-P50 (K71) (68.4)	β3β4 loop	α-K39 (120.4); α-K38 (38.5)
t-S53† (A74) (25.4)	β3β4 loop	α-K39† (120.4)
t-K58† (−) (17.8)	β3β4 loop	α-Q18† (79.4)
t-T69 (Y89) (10.1)	β4	
t-K70 (Y90) (39.6)	β4	
t-Q73† (C93) (78.3)	β4	β-Y60† (57.3); β-B64† (37.8)
t-H74 (T94) (36.6)	β4	
t-T75† (F95) (54.5)	β4	p-A13† (61.6)
t-S76† (S96) (14.7)	β4β5 loop	p-A13† (61.6)
t-E77 (K97) (21.7)	β4β5 loop	
t-I81 (K111) (21.5)	β5	
t-F83 (C113) (15.3)	β5	α-V65 (37.3)
t-I85 (Y115) (12.8)	β5	
Other residues at the contact region with more than 4 Å separation from the TSST-1 residues		α-I72 (25.2) p-A7 (13.6) p-A10 (45.8)

*van der Waals contact (<~3.8 Å). †Potential hydrogen bond (<~3.5 Å). ‡Potential salt bridge.

Fig. 3. Location of TSST-1 residues reported to be involved in TCR interactions. The α1 and β1 domains of DR1 are in light blue and blue, the antigenic peptide is yellow, and TSST-1 is red. Residues t-Y115, t-E132, t-H135, t-I140, t-H141, and t-Y144, which are important for mitogenic activity of TSST-1 (*26*) and have been implicated in TCR binding (*9*, *10*), are represented as ball-and-stick models.

reveals that 11 of the 17 DR1 residues in the TSST-1:DR1 interface (underlined in Table 3) are common to the SEB:DR1 interface, despite the overall difference in orientations of the two superantigens on DR1. (Thirteen of the 19 positions on TSST-1 that contact DR1 are homologous to positions on SEB that contact DR1.) Thus, it seems impossible for TSST-1 and SEB to bind simultaneously to DR1, as they would need to occupy the same space. Yet, neither TSST-1 nor SEB appears to be able to completely inhibit the binding of the other (*11*, *13*, *25*). One possible explanation for this dilemma would be the existence of a second binding site on DR1 for SEB or TSST-1, but there is no evidence for such a site.

The most striking new observation about superantigen–class II interaction seen in the TSST-1:DR1 complex is that the superantigen covers most of the peptide binding site, contacting all the polymorphic residues on the α chain α helix, residues on the bound peptide, and part of the β chain α helix, across the peptide binding site. This contrasts with our expectation because superantigen activation of T cells is reportedly much less MHC-restricted and peptide-dependent than peptide antigen–induced activation. It suggests that TSST-1 binding to DR1 may be in part peptide-dependent. Recent binding measurements between superantigens and DR molecules on different cell types suggest that different subsets of HLA-DR molecules may bind TSST-1 and SEB (*25*). Peptide-dependent binding offers a possible mechanism for superantigens to distinguish different subsets of DR1 molecules and that in turn could account for the inability of TSST-1 to inhibit completely the binding of SEB to DR1. Superantigen activation dependent on peptide (and hence also MHC allele) would allow a pathogen to direct T cell activation by its antigens or by host antigens during infections, with potential consequences for inducing specific autoreactivity.

REFERENCES AND NOTES

1. P. Marrack, M. Blackman, E. Kushnir, J. Kappler, *J. Exp. Med.* **171**, 455 (1990).
2. Y. Choi *et al.*, *ibid.* **172**, 981 (1990).
3. B. Fleischer and H. Schrezenmeier, *ibid.* **167**, 1697 (1988); P. Marrack and J. Kappler, *Science* **248**, 705 (1990).
4. A. M. Pullen, J. Bill, R. T. Kubo, P. Marrack, J. W. Kappler, *J. Exp. Med.* **173**, 1183 (1991); B. Fleischer and H.-W. Mittrücker, *Eur. J. Immunol.* **21**, 1331 (1991); A. Seth *et al.*, *Nature* **369**, 324 (1994).
5. R. M. Germain and D. H. Margulies, *Annu. Rev. Immunol.* **11**, 403 (1993).
6. A. M. Pullen, T. Wade, P. Marrack, J. W. Kappler, *Cell* **61**, 1365 (1990); Y. Choi *et al.*, *Nature* **346**, 471 (1990).
7. T. S. Jardetzky *et al.*, *Nature* **368**, 711 (1994).
8. P. Dellabona *et al.*, *Cell* **62**, 1115 (1990); J. L. Jorgensen, P. A. Reay, E. W. Ehrich, M. M. Davis, *Annu. Rev. Immunol.* **10**, 835 (1992).
9. G. S. Prasad *et al.*, *Biochemistry* **32**, 13761 (1993).
10. K. R. Acharya *et al.*, *Nature* **367**, 94 (1994); S. Swaminathan, W. Furey, J. Pletcher, M. Sax, *ibid.* **359**, 801 (1992).
11. P. R. Scholl, A. Diez, R. S. Geha, *J. Immunol.* **143**, 2583 (1989).
12. J. D. Fraser, *Nature* **339**, 221 (1989).
13. M. M. Chintagumpala, J. A. Mollick, R. R. Rich, *J. Immunol.* **147**, 3876 (1991).
14. P. Panina-Bordignon, X.-t. Fu, A. Lanzavecchia, R. W. Karr, *J. Exp. Med.* **176**, 1779 (1992).
15. J. Thibodeau, N. Labrecque, F. Denis, B. Huber, R.-P. Sékaly, *ibid.* **179**, 1029 (1994).
16. J. H. Brown *et al.*, *Nature* **364**, 33 (1993); L. J. Stern *et al.*, *ibid.* **368**, 215 (1994).
17. P. J. Bjorkman *et al.*, *ibid.* **329**, 506 (1987); M. A. Saper, P. J. Bjorkman, D. C. Wiley, *J. Mol. Biol.* **219**, 277 (1991).
18. A. T. Brünger, *J. Mol. Biol.* **203**, 803 (1988).
19. C. Edwin *et al.*, *J. Infect. Dis.* **163**, 524 (1991); J. M. Soos, J. K. Russell, M. A. Jarpe, C. H. Pontzer, H. M. Johnson, *Biochem. Biophys. Res. Commun.* **191**, 1211 (1993).
20. B. Lee and F. M. Richard, *J. Mol. Biol.* **55**, 379 (1971).
21. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
22. J. H. Brown *et al.*, *Nature* **332**, 845 (1988).
23. K. R. Hudson, S. C. Lowe, R. G. Urban, J. D. Fraser, personal communication.
24. D. R. Karp, C. L. Teletski, P. Scholl, R. Geha, E. O. Long, *Nature* **346**, 474 (1990); N. S. Braunstein, D. A. Weber, X. C. Wang, E. O. Long, D. Karp, *J. Exp. Med.* **175**, 1301 (1992).
25. J. Thibodeau *et al.*, *Science* **266**, 1874 (1994).
26. L. Blanco *et al.*, *Infect. Immun.* **58**, 3020 (1990); P. F. Bonventre, H. Heeg, C. Cullen, C. J. Lian, *ibid.* **61**, 793 (1993); D. L. Murray *et al.*, abstract, 93rd General Meeting of the American Society of Microbiology (Atlanta, GA, 1993), vol. 64.
27. J. C. Gorga, V. Horejsi, D. R. Johnson, R. Raghupathy, J. L. Strominger, *J. Biol. Chem.* **262**, 16087 (1987). Water-soluble human lymphocyte antigen–DR1 (HLA-DR1) molecules (DRA and DRB1*0101) were immunoaffinity-purified from the lymphoblastoid cell line LG-2 and digested with papain in a manner similar to that described by Gorga *et al.* (above). Detergent-soluble DR (10 mg) was digested with papain, and after iodoacetamide quenching the DR1-papain mixture was dialyzed 12 hours at 4°C against 50 volumes of deionized water. The dialyzed mixture was then transferred to pre-chilled ultracentrifugation tubes and subjected to 35,000*g* for 1 hour at 4°C. The cleared supernatants were then adjusted to 50 ml containing a 2% final concentration of ampholytes (Ampholine, 3.5-10, Pharmacia LKB, Uppsala, Sweden). The mixture was then transferred into a preparative isoelectric focusing cell (Rotofor, Bio-Rad Laboratories, Richmond, CA) and focused at 12 W for 5 hours at 4°C. Fractions were then collected, and those containing DR (pH 4.0 to 5.5) were pooled and dialyzed against 50 volumes of 10 mM tris-HCl (pH 7.5). The sample was then concentrated to 2 ml by vacuum dialysis against 10 mM tris-HCl (pH 7.5) and further purified by size exclusion chromatography (SEC-3000; 5 μm; 7.5 cm by 30 cm, Beckman Instruments). The DR peak was collected and reconcentrated to >10 mg/ml by vacuum dialysis as above.
28. W. Kabsch, *J. Appl. Crystallogr.* **21**, 916 (1988).
29. G. N. Reeke, *ibid.* **17**, 125 (1984); D. H. Bethe, *ibid.*, p. 215.
30. B. C. Wang, *Methods Enzymol.* **115**, 90 (1985).
31. T. A. Jones, *ibid.*, p. 151.
32. J. P. Kraulis, *J. Appl. Crystallogr.* **24**, 946 (1991).
33. Another contact is found in the crystal between a symmetrically related *TSST-1 molecule and the TSST-1:DR1 complex. Of the six TSST-1 residues implicated in TCR recognition by mutagenesis (Y115, E132, H135, I140, H141, and Y144) (*26*), two (Y115 and Y144) are in the TSST-1:*TSST-1 contact and three (H135, I140, and H141) are in its immediate vicinity.
34. We thank D. H. Ohlendorf, J. H. Brown, and L. J. Stern for the coordinates of TSST-1 and DR1; N. Ramesh and R. S. Geha for an initial sample of purified TSST-1; P. Klimovitsky and A. Haykov for technical assistance; and M. Pietras for large-scale production of tissue culture cells. Discussions with T. S. Jardetzky, D. N. Garboczi, and A. Seth and help from D. C. Rees, P. J. Bjorkman, S. E. Ryu, M. J. Eck, R. S. Brown, R. Nolte, and C. Garnett are appreciated. J.K. acknowledges support by the Howard Hughes Medical Institute (HHMI) and NIH. R.G.U. is supported by the Irvington Institute for Medical Research. D.C.W. is an investigator of HHMI. Research supported by an NIH grant to D.C.W. Coordinates will be deposited in the Protein Data Bank and are available before their release by e-mail (kim@xtal0.harvard.edu).

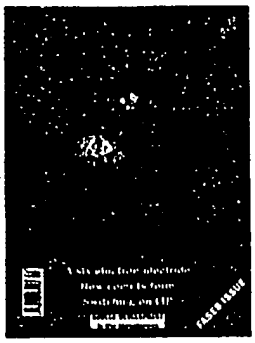
11 July 1994; accepted 19 October 1994

Subsets of HLA-DR1 Molecules Defined by SEB and TSST-1 Binding

Jacques Thibodeau, Isabelle Cloutier, Pascal M. Lavoie, Nathalie Labrecque,* Walid Mourad, Theodore Jardetzky,† Rafick-Pierre Sékaly‡

Superantigens bind to major histocompatibility complex class II molecules on antigen-presenting cells and stimulate T cells. *Staphylococcus aureus* enterotoxin B (SEB) and toxic shock syndrome toxin–1 (TSST-1) bind to the same region of human lymphocyte antigen (HLA)–DR1 but do not compete with each other, which indicates that they bind to different subsets of DR1 molecules. Here, a mutation in the peptide-binding groove disrupted the SEB and TSST-1 binding sites, which suggests that peptides can influence the interaction with bacterial toxins. In support of this, the expression of the DR1 molecule in various cell types differentially affected the binding of these toxins.

Superantigens (SAGs) are T cell mitogens produced by a variety of bacteria and viruses (*1*). The formation of a trimolecular complex between SAGs, major histocompatibility complex (MHC) class II molecules, and the T cell receptor (TCR) leads to the activation of T cells in a V_β-restricted fashion (*2*). The most studied superantigens of



nature

21 April 1994

Vol. 368 Issue no. 6473

◀ Superantigens are implicated in a number of diseases and in autoimmunity, so the understanding of their interactions with the immune system is of broad interest. The three-dimensional structure of a bacterial superantigen bound to a human class II histocompatibility complex molecule is reported on page 711. The antigen binds as an intact protein outside the 'normal' peptide antigen binding site of the class II MHC molecule.

THIS WEEK . . . THIS WEEK . . . THIS WEEK . . .

Electron rings

Electrides are crystalline salts containing alkali metal ions and trapped electrons to balance the metal's charge. Only four such compounds have so far been structurally characterized, number four being described on page 726. The new compound confirms that electrides are a varied group. A central six-oxygen crown ether is surrounded by six caesium cations, each one sandwiched between a further two crown ethers. The six 'balancing' electrons seem to be trapped in a puckered ring above and below the central molecule.

Making comets

The spectacular disintegration of the Jupiter-bound comet Shoemaker–Levy 9 into about 20 fragments supports the view that cometary nuclei are piles of 'rubble' loosely held together by mutual gravitational attraction. How are these fragile structures formed in the outer Solar System? Processes involving either collisional coagulation in the solar nebula or gravitational collapse of a layer of dust particles have been proposed. Now Weidenschilling shows that a two-stage process involving elements of both models provides a better explanation for the observed cometary structures. Pages 721 and 687.

LTP switch

Long-term potentiation (LTP) in the hippocampus is a popular model for the synaptic processes learning and memory. A new feature of LTP is reported on page 740, a 'switch' mechanism in which LTP is activated by metabotropic glutamate receptors.

Eye problem

The apparent complexity and sheer improbability of an organ as specialized as the vertebrate eye may seem to constitute a challenge to the mechanism of evolution. But new simulations suggest that a fish eye could have evolved step-by-step from flat skin in a mere 400,000 years. Page 690.

Methane from the deep

Surface ocean waters are typically supersaturated with the greenhouse gas methane but the origin of the methane is unclear, mainly because methane producers need anaerobic environments but the surface water is well oxygenated. Karl and Tilbrook (page 732) offer a solution to this 'oceanic methane paradox', showing that methane produced by bacteria in the reducing environment provided by sinking particles of organic matter can produce the observed methane supersaturations.

Kidney disorders

Renal cysts account for some 10% of kidney transplant dialysis patients, and effective treatments for the condition have proved elusive. But tests in an *in vitro* model for cyst formation and in a mouse model for congenital polycystic kidney disease show that taxol can inhibit cyst formation and prevent uraemic death. Page 750.

Ecosystem threat

More evidence that the loss of biodiversity in the wake of human expansion has a deleterious effect on the continued survival of whole ecosystems. Naeem *et al.* reveal how experimental manipulation of biodiversity in closed, regulated ecological microcosms shows that biogeochemical processes in these systems can be increasingly altered or damaged by reduced biodiversity. Pages 734 and 686.

Cancer causation

Cyclin-dependent kinase-4 inhibitor gene is identified as a likely candidate for the cytogenetic disruption of chromosome 9p21, which is associated with malignant melanomas, gliomas, lung cancers and leukaemias. In this issue, Nobori *et al.* report the positional cloning of this vulnerable gene which may inhibit cell proliferation in its normal guise. Page 753.

Guide to Authors

Page 778.

NATURE SAYS

NATURE REPORTS

CORRESPONDENCE

COMMENTARY

NEWS AND VIEWS

SCIENTIFIC CORRESPONDENCE

BOOK REVIEWS

Nature® (ISSN 0028-0836) is published weekly on Thursday, except the last week in December, by Macmillan Magazines Ltd (4 Little Essex Street, London WC2R 3LF). Annual subscription for USA and Canada US$425 (institutional/corporate), US$135 (individual making personal payment). US and Canadian orders to: *Nature*, Subscription Dept, PO Box 1733, Riverton, NJ 08077-7333, USA. Other orders to *Nature*, Brunel Road, Basingstoke, Hants RG21 2XS, UK. Second class postage paid at New York, NY 10012, and additional mailing offices. Authorization to photocopy material for internal or personal use, or internal or personal use of specific clients, is granted by *Nature* to libraries and others registered with the Copyright Clearance Centre (CCC) Transactional Reporting Service, provided the base fee of $9.00 an article (or $1.00 a page) is paid direct to CCC, 27 Congress Street, Salem, MA 01970, USA. Identification code for *Nature*: 0028-0836/94 $9.00 + $1.00. US Postmaster send address changes to: Nature, 65 Bleecker Street, New York,

Thr -dim nsi nal structur f a human class II histoc mpatibility mol cul c mplexed with superantigen

Th d re S. Jardetzky, Jerry H. Brown*†, Joan C. Gorga‡, Lawr nce J. Stern*, Robert G. Urban, Young-in Chi§, Cynthia Stauffacher§, Jack L. Strominger & Don C. Wiley*‖

Department of Biochemistry and Molecular Biology, * Howard Hughes Medical Institute, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA
† Rosenstiel Research Center, Brandeis University, 415 South Street, Waltham, Massachusetts 02154, USA
‡ Department of Pediatrics, Children's Hospital of Pittsburgh, 6130 Rangos Research Building, 3705 Fifth Avenue, Pittsburgh, Pennsylvania 15213, USA
§ Department of Biology, Purdue University, West Lafayette, Indiana 47907, USA

The structure of a bacterial superantigen, *Staphylococcus aureus* enterotoxin B, bound to a human class II histocompatibility complex molecule (HLA-DR1) has been determined by X-ray crystallography. The superantigen binds as an intact protein outside the conventional peptide antigen-binding site of the class II major histocompatibility complex (MHC) molecule. No large conformational changes occur upon complex formation in either the DR1 or the enterotoxin B molecul s. The structure of the complex helps explain how different class II molecules and superantigens associate and suggests a model for ternary complex formation with the T-cell antigen receptor (TCR), in which unconventional TCR–MHC contacts are possible.

SUPERANTIGENS comprise a class of disease-associated, immunostimulatory molecules that bind class II MHC molecules and stimulate large numbers of T cells[1,2]. Members of the superantigen family include toxins from *S. aureus* and other bacteria[3], as well as viral superantigens from mouse mammary tumour virus (MMTV)[4]. The *S. aureus* toxins are associated with food poisoning and toxic-shock syndrome, and the MMTV superantigen plays a critical role in viral transmission. The toxicity of bacterial superantigens is thought to be mediated by their potent T-cell-stimulating activities[5], leading to lymphokine release[6], respiratory distress and shock. Superantigens have also been implicated in rabies, rheumatoid arthritis, and mouse and human AIDS[7].

The mechanism by which superantigens stimulate T cells differs from that of normal antigens. Conventional T-cell antigens are short proteolytic peptides from foreign proteins, bound in the peptide-binding groove of class I or class II MHC molecules[8]. The structures of both class I and class II MHC molecules[9–11] demonstrate that these bound peptides become an integral part of the MHC protein surface, which is displayed by antigen-presenting cells to specific T cells, generating an immune response. In contrast, bacterial superantigen activity is abolished by proteolysis and it is the intact superantigen protein that interacts with class II MHC molecules outside the peptide-binding groove[12,13] in order to stimulate T cells.

The interaction of conventional peptide antigens and superantigens with the T-cell antigen receptor (TCR) also differs. TCR molecules are structurally related to antibody molecules, with hypervariable regions forming a combining site for a specific peptide–MHC combination[12]. Superantigens bypass this specificity-determining region of the TCR, and interact with a surface of the TCR predicted to lie outside the antigen-combining site on the variable β-chain (Vβ) domain[14–18]. This ability of superantigens to interact with both MHC and TCR molecules outside their normal antigen-specific sites leads to the stimulation of many more T cells than observed with normal peptide antigens.

To understand better the molecular basis of the pathological effects of superantigens, we have determined the structure of a bacterial superantigen, *S. aureus* enterotoxin B (SEB), bound to a human class II MHC molecule, HLA-DR1, by X-ray crystallography. SEB binds outside the MHC peptide-binding site, with the N-terminal domain of SEB interacting with the DR1 α1 domain. No large conformational changes are observed in either the class II molecule[11] or the SEB molecule[19] upon complex formation. SEB residues that affect the interaction with T-cell receptors are positioned to the side and above the DR1 peptide-binding site of the class II molecule, suggesting a model for the interaction of superantigen–MHC complexes with TCR, where normal TCR–MHC interactions are blocked.

Structure determination

The structure of the DR1–SEB complex was determined using the data sets listed in Table 1, from two different DR1–SEB crystal forms. Initial electron density maps were generated using SIR/anomalous phases for crystal form I and improved by averaging with maps derived from HLA-DR1 crystals as described[11]. The initial model of the DR1 molecule was improved by cycles of building and refinement and a partial model for the SEB was built. A low-resolution data set of crystal form II (Table 1) was solved by molecular replacement with the partial model of the complex, and iterative non-crystallographic four-fold symmetry averaging was used (Fig. 1*a*) to obtain a polyalanine trace for 190 residues of the SEB molecule at low resolution. This model was used to calculate higher-resolution electron-density omit maps, followed by iterative non-crystallographic two-fold symmetry averaging, with data from crystal form I, and improved by further cycles of building and refinement (Fig. 1*b*).

‖ To whom correspondence should be addressed.

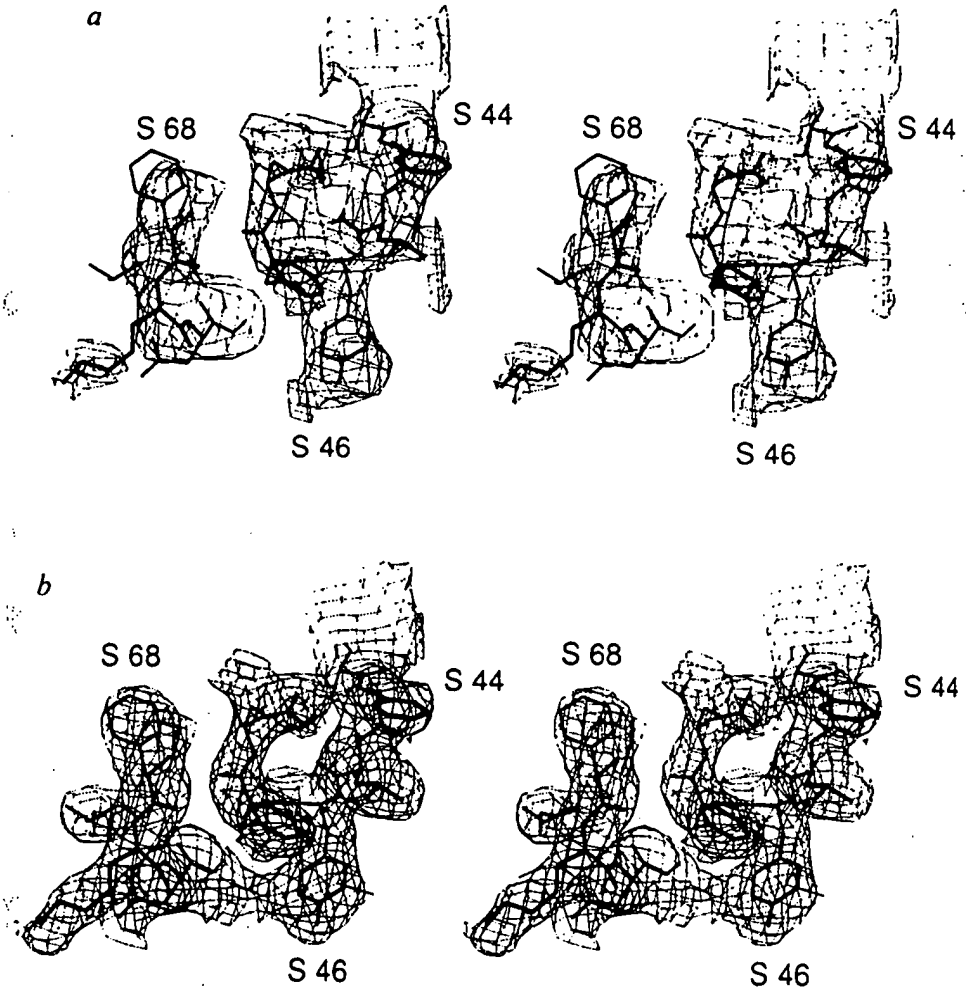
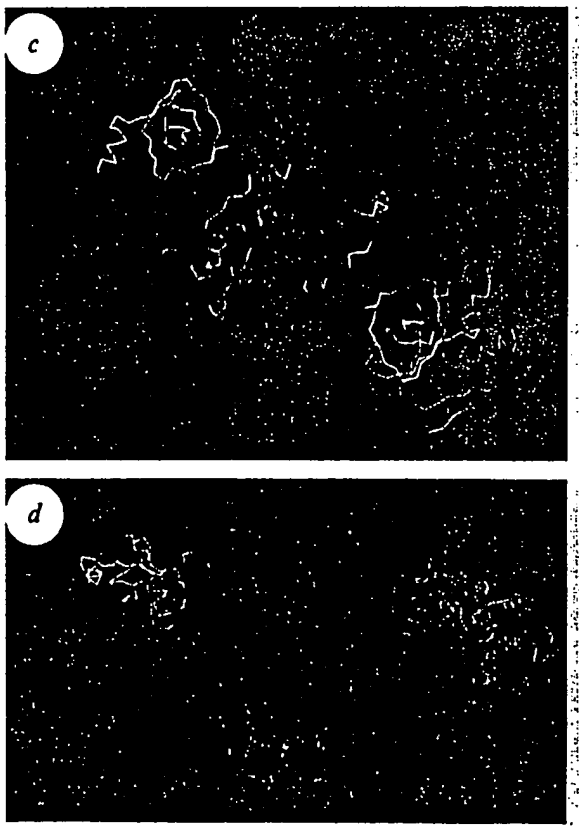


FIG. 1 Electron density maps and temperature factors for the DR1–SEB complex. SEB electron density maps at the DR1–SEB interface. *a*, 15.0–4.3 Å $|2F_o - F_{calc}|$ map generated by 4-fold iterative averaging between crystal form I and form II. *b*, A current 2-fold averaged $|2F_o - F_{calc}|$ omit map at 2.7 Å resolution, calculated using current model phases and data from crystal form I, omitting the atoms shown. Both maps are contoured at 1.0σ with a cover radius of 1.5 Å around the atoms shown. *c* and *d*, Top and side views (respectively) of the DR1–SEB complex showing the radial increase in temperature factor. Two DR1–SEB complexes are found in the asymmetric unit as shown. The current model is coloured by temperature factor (atomic *B*-factor), with blue representing Cα *B*-factor values less than 25 Å^2 and red representing *B*-factor values greater than 100 Å^2 (see Table 1 for average SEB *B*-factors). Note the increase in *B*-factor as a function of distance from the DR1–SEB interface, and the correspondence with loops in the DR1 structure. The high-temperature factors may be due to SEB disorder within the crystal lattice. One SEB makes no contacts with other symmetry-related molecules in the lattice, whereas the other SEB molecule has only few crystal contacts in regions of the C-terminal domain that may not be accurately modelled. The DR1 molecules form crystal contacts in all lattice directions and may provide the predominant stabilization of the crystal lattice. The high-temperature factors may therefore also reflect a lower SEB occupancy within the lattice. Refinement of SEB occupancy before *B* refinement typically provides an improvement in R_{free} of 0.3–0.5%. Further experiments are necessary to resolve these possibilities.



The structure of the complex shows a gradient of disorder of the SEB molecules, extending radially out from the DR1–SEB interface (Fig. 1*c*, *d*). This has two consequences. First, five surface loops of the SEB molecules show no interpretable electron density, presumably because of the higher basal level of SEB disorder. Second, the SEB C-terminal domain shows generally weaker electron density, with stunted or absent side-chain density. The N-terminal domain of the SEB molecule, which forms most of the contacts to DR1 (Fig. 1*b*; and shown in yellow in Fig. 2*a*) is, however, the best ordered region of the SEB structure. The disorder evident in the structure does not affect either our major conclusions as to how SEB binds to class II MHC molecules or the implications for TCR interactions.

Overview of DR1–SEB complex formation

Figure 2 shows top and stereo views of the DR1–SEB complex. SEB only contacts residues of the α1 domain of DR1, interacting with amino acids from the first and third turns of the β-sheet and from the N-terminal region of the α-helix. These residues form a deep, concave surface to one side of the peptide-binding site of DR1 (Fig. 3*c*), in agreement with mutational studies mapping the MHC–SEB interaction[13]. The potential influence of α-chain polymorphisms in the binding of SEB to other human and mouse class II molecules is discussed later. SEB does not interact

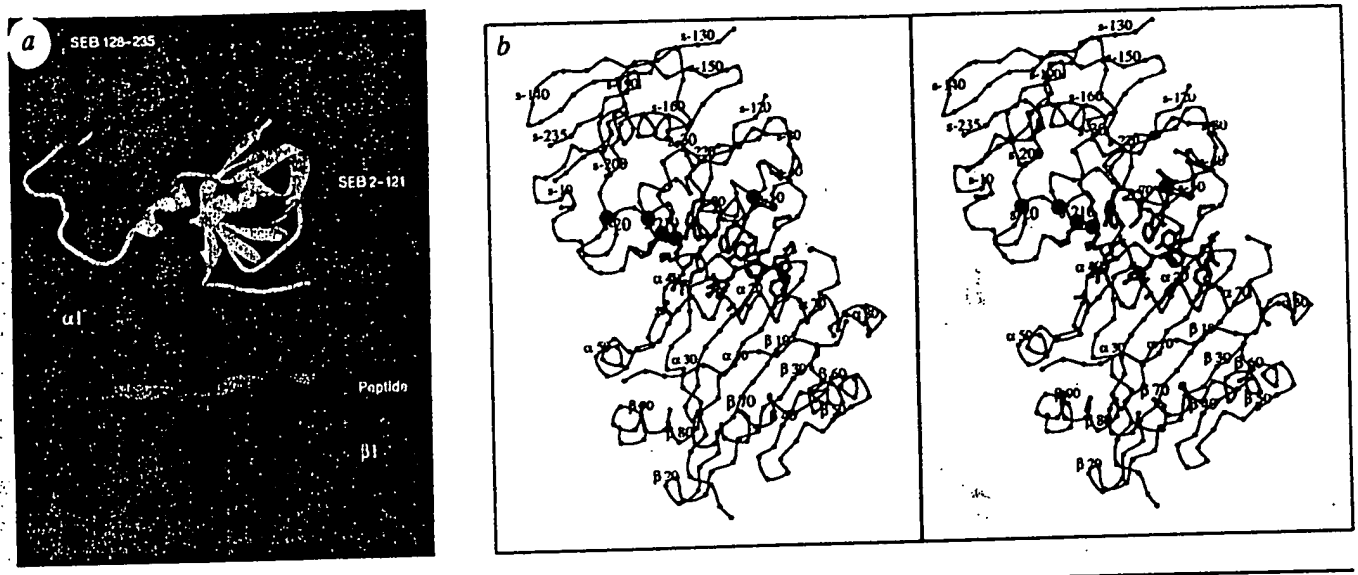
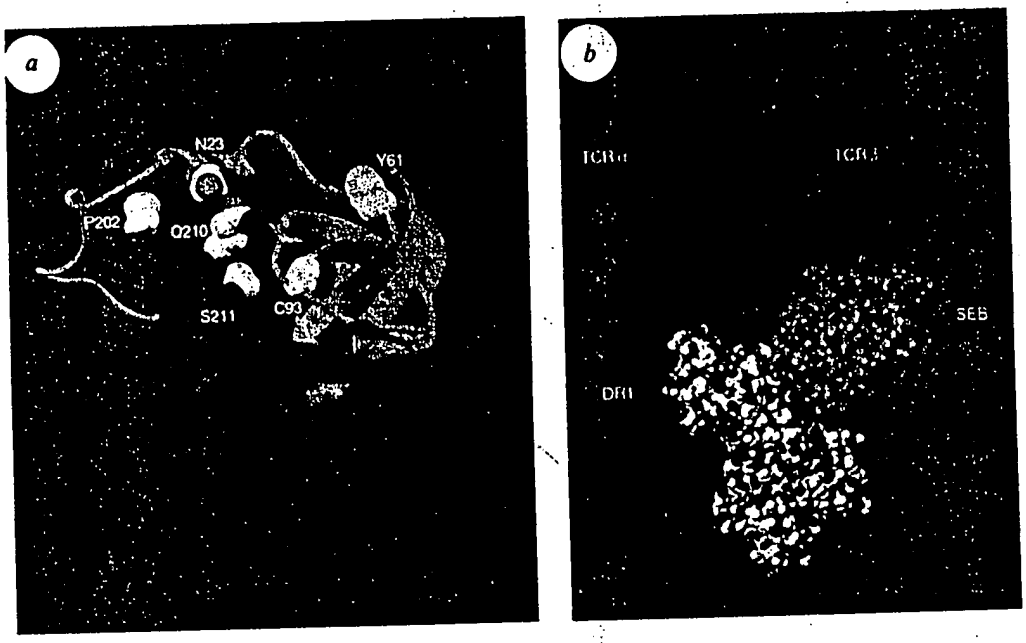


FIG. 2 Overview of the complex between HLA-DR1 and SEB. *a*, Top view, HLA-DR1 α1 and β1 domains are shown in blue; α2 and β2 domains are not shown. N-terminal residues of SEB 2–121 are in yellow, and C-terminal residues 127–235 of SEB are in red; the peptide is shown in pink. The peptide conformation is based on fitting a polyalanine chain into the observed electron density corresponding to a mixture of self-peptides bound to the HLA-DR1 molecule. *b*, Cα trace of the complex (DR1 α2 and β2 not shown), showing all side chains involved in the DR1–SEB interface. SEB interface residues are in yellow; DR1 interface residues are in dark blue. Red spheres mark the SEB and SEA residues implicated in TCR interactions[2,24,41–43].



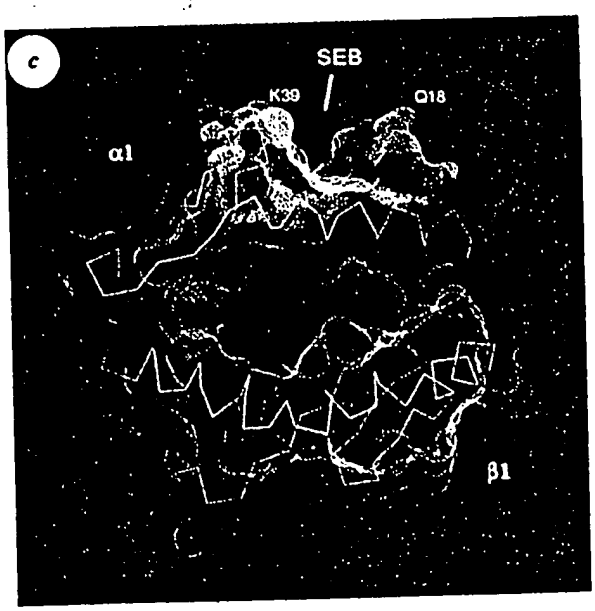


FIG. 3 Location of superantigen residues involved in TCR interactions and a model of ternary complex formation between DR1, SEB and TCR. *a*, Residues in SEB (N23, Y61, C93–C113; N60 not shown) and SEA (in SEA: G200, S206, N207; in SEB P202, Q210, S211) that have been implicated in TCR interactions by mutagenesis[2,24,41–43]. DR1 α1 and β1 domains are blue (DR1 α2 and β2 domains are not shown), SEB is yellow (N-terminal residues) and red (C-terminal residues), and peptide is pink. CPK representation is white for carbon atoms, red for oxygen, blue for nitrogen, and green for sulphur. *b*, Hypothetical model of ternary complex formation of DR1 and SEB with TCR. An immunoglobulin Fab fragment model of the TCR α-chain in blue, β-chain in red. Hypervariable regions of TCR (CDR1, CDR2 and CDR3) are shown in yellow, and the HV4 loop and *B* strand of the Vβ domain are shown in white. DR1 carbon atoms are white, SEB carbon atoms are yellow, peptide carbon atoms are magenta. The view is looking down the DR1 peptide-binding site, with the DR1 β-chain to the left and the α-chain to the right. The TCR is positioned so that the SEB residues shown in *a* can interact with the white HV4 loop. *c*, Top view of the class II molecule, showing the region of the α1 domain buried by complex formation with SEB. Surface of the DR1 molecule shown in magenta outside the SEB interaction area. Blue, hydrophobic surface buried by SEB: yellow, polar surface buried by SEB.

with class I MHC molecules and this region differs in class I and class II MHC structures[11]. Good peptide density is observed in the peptide-binding site, corresponding to a mixture of self-peptides, and has been modelled as polyalanine (Fig. 2). SEB does not interact directly with the peptide and the peptide conformation is very similar to that observed for a single peptide–DR1 complex[20].

Residues from the SEB molecule that interact with DR1 derive predominantly from the smaller N-terminal β-barrel domain of the SEB molecule, although three residues from the C-terminal helix 5 (residues 210–217) also contact the DR1 molecule. Helix 5 is actually more closely associated with the N-terminal domain than with the C-terminal domain (Fig. 2*b*). The SEB residues most central to the interface lie in a turn between strands 1

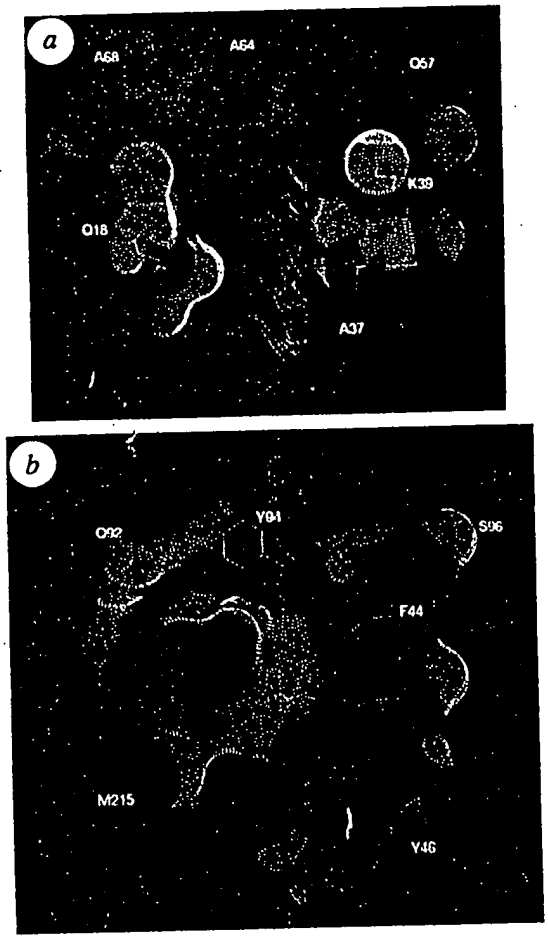


FIG. 4 The DR1–SEB binding interface. *a*, Surface view of the DR1 residues involved in binding to SEB. Yellow, polar atoms at surface; blue, hydrophobic atoms at surface. *b*, Surface view of the SEB residues involve in binding, same colour scheme as in *a*. *c*, DR1 residues involved complex formation, side view. DR1 residues are shown with blue bonds. Red, green and dark blue spheres represent oxygen, sulphur and nitrogen atoms, respectively. *d*, SEB residues involved in complex formation. SEB residues are shown with yellow bonds and atoms coloured as in *c*. *e*, Salt bridge formed between DR1 α-chain residue lysine 39 and SEB glutamic acid 67, with SEB tyrosines 89 and 115 forming hydrogen bonds. *f*, Potential hydrogen bonds formed between residues of the DR1 molecule and the main chain of residues 43–46 of the SEB molecule. Surfaces generated with MS[23] using a probe radius of 1.4 Å, and displayed in O[64]; *c–f* generated with Molscript[65]. Surfaces areas quoted in the text were calculated with the default atomic radii used by the program Access[22] and a probe radius of 1.4 Å.

(residues 33–39) and 2 (residues 48–52) and along strand 3 (residues 63–68). In addition, a stretch of residues in the SEB disulphide loop (residues 92–96) runs above the binding interface parallel to the DR1 α1 α-helix. The C-terminal domain of the SEB molecule is oriented up and away from the class II molecule, consistent with the observation that N-terminal constructs containing residues 1–138 of the SEB molecule bind class II MHC molecules and retain partial activity[21].

Description of the Interface

Figure 2*b* shows a stereo view of the DR1 and SEB residues involved in complex formation. The interface is comparable in size to antigen–antibody interfaces, burying 780 Å^2 and 760 Å^2 of the DR1 and SEB solvent-accessible[22] surfaces respectively. Twenty-one residues of the DR1 molecule and nineteen residues from the SEB molecule are involved in complex formation.

A topological view of the binding interface shows a dramatic division of the complementary surfaces of the DR1 and SEB molecules. Figure 4*a*, *b* shows the molecular surfaces[23] of the DR1 and SEB molecules respectively, which are buried upon formation of the complex (coloured blue for non-polar atoms and yellow for polar atoms). Each binding surface is divided into two regions, one predominantly hydrophobic and one predominantly polar.

The hydrophobic region of the interface consists of a ridge of non-polar residues (F44, L45 and F47 (single-letter amino-acid code with residue number)) protruding from the loop between strands 1 and 2 of the SEB molecule (Fig. 4*b*, blue; and Fig. 4*d*), which fits into a predominantly hydrophobic depression on the DR1 molecule formed by residues of loops 1 and 3 and the α-helix of the α-chain (Fig. 4*a*, blue; Figs 3*c* and 4*c*). DR1 residues that contribute to this interaction include hydrophobic residues Y13, M36, A37, L60, I63 and A64, as well as the aliphatic portions of more polar residues such as Q18 (Fig. 4*c*). Mutations of residues F44 and L45 in the SEB molecule disrupt binding to class II MHC molecules[24].

The polar region of the binding interface is more apparent on the SEB interaction surface, to the left of the hydrophobic ridge (Fig. 4*b*). This polar pocket on the SEB surface is complemented by a protrusion from loop 3 of the DR1 molecule formed by lysine 39 (Fig. 4*a*, *e*). This lysine forms a completely buried salt bridge with glutamic acid at position 67 of SEB, surrounded by hydrogen bonds from SEB Y89 and Y115 (Fig. 4*e*). The mutation of lysine 39 to alanine in the DR1 molecule reduces SEB binding[40].

Three other residues of HLA-DR1 (Y13, Q18 and K67) are potentially involved in hydrogen bonds to the main chain of SEB residues 43–46 (Fig. 4*f*) and may be important in positioning the hydrophobic residues of the SEB molecule between the DR1 loops. As these residues vary between class II isotypes (Fig. 5), they may play a part in determining differences in the overall binding affinity.

In addition, SEB disulphide loop residues 92–96 contact the α1-domain α-helix of the DR1 molecule along the upper face of the interaction region (Fig. 4*d*). In particular, SEB Y94 forms an extensive set of hydrophobic interactions with DR1 residues 60 and 61, whereas SEB S96 contacts DR1 residues 64, 67 and 68. Although these SEB residues do not bind into distinct pockets on the DR1 molecule, they have important implications for the accessibility of DR1 residues to TCR interactions (see below).

Two SEB residues (14 and 17) have been implicated in MHC binding[24], but are not directly involved in the DR1–SEB interface and may have an indirect effect on MHC binding. This conclusion is supported by the observation that deletion of SEB residues 1–30 does not abrogate the ability of SEB to stimulate polyclonal T cells[21].

Other class II molecules and SEB

The binding affinity of SEB varies between different class II MHC molecules. The ability of SEB to bind many different DR allotypes[25] can be explained by its exclusive interaction with the DR1 α-chain, which is conserved in all DR molecules. Binding to other class II isotypes is weaker than binding to DR[26–29] in the order DR > DQ > DP[25,29], whereas for mouse alleles I-E binds SEB better than I-A[29], but both bind more weakly than DR.

Figure 5*a* shows a plot of the surface of the DR1 molecule that is buried by the interaction with SEB, along with the corresponding residues that are found in other human (DP/DQ) and mouse (I-A/I-E) alleles. For the human class II isotypes DP and DQ, about 50% of the residues in the DR1–SEB interface are conserved, although the subset of these residues differs between DP and DQ. Lysine 39 is found in all three isotypes, indicating that a salt bridge with E67 of SEB could be formed (Fig. 4*e*). Many of the residues that form the hydrophobic portion of the interaction surface (Fig. 4*c*, and shown in blue in Fig. 4*a*) are conserved (L60, A64) or conservatively substituted (M36 to L, I63 to I or M).

Although a number of residue differences could account for a lower binding affinity for DQ and DP, relative to DR, the substitution of Q18 to proline in both DQ and DP is particularly central to the DR1–SEB interface. Proline would disrupt one hydrogen bond (Fig. 4*e*), and would potentially alter the conformation of the other residues in this loop. DP molecules have additional mutations in residues in this region (Y13 to valine and K67 to asparagine) that form one side of the SEB binding site (Figs 3*c* and 4*a*, *b*), which could further destabilize the interaction with SEB molecules.

In the case of the mouse class II molecules, 12 of 17 residues are conserved in I-E molecules and 10 of 17 in I-A molecules. I-E molecules have a lysine-to-serine mutation at position 39, which would abolish the salt bridge with SEB (Figs 4*e* and 5*a*). Serine may partially compensate for the lost salt-bridge interaction. Further mutational studies are needed to define the function of different amino acids at the interface.

Other superantigens and class II molecules

Although the sequence similarity of different *S. aureus* toxins with SEB ranges from 40 to 90%, there is evidence for distinct MHC binding sites for different toxins[28,30,31]. Binding of *S. aureus* enterotoxin A (SEA) has been mapped to the MHC class II β1 domain[32,33]; binding of toxic-shock-syndrome toxin TSST-1 has been shown to be sensitive to both α1 and β1 domains[34–36]. A comparison of the SEB residues involved in binding to DR1 with the corresponding residues in SEA and TSST-1 provides some insight into the functional data available for these toxins.

Figure 5*b* shows a plot of the buried surface area of the SEB residues involved in binding DR1, with the corresponding residues from other *S. aureus* toxins. Of the DR1–SEB interactions described above, a number of central residues are conserved or conservatively substituted in SEA, including F44, L45, D67, Y89 and Y115. The conservation of these residues suggests that SEA may bind to class II molecules in a similar way to SEB. Substitution of the amino acids corresponding to SEB residues F44 and L45 in SEA (F47, L48) reduces its T-cell-stimulatory activity[37] and substitution of SEA L48 reduces MHC binding but does not abolish it (J. Kappler, personal communication). Other interactions discussed below may contribute to additional SEA binding.

Figure 5*b* shows that the major features of the SEB-binding interface are absent in TSST-1, based on a structural alignment of the two proteins[38,39]. These changes include the loss of the hydrophobic ridge (F44 to S) and the residues that interact with DR1 K39 (SEB E67 to I, Y89 to T, Y115 to I). Mutation of α-chain residues M36 to I, or K39 to S, abolishes TSST-1 binding to HLA-DR7 (ref. 35). Both of these residues are directly involved in the DR1–SEB binding interface (Fig. 4) and mutation of K39 to A disrupts SEB binding as well as TSST-1

binding[40]. This indicates that although SEB and TSST-1 are sensitive to mutations in the same region of the class II α-chain, their specific interactions may be substantially different.

Superantigen residues interacting with TCR

Some mutations in the SEB molecule affect T-cell stimulation, but not class II binding, suggesting specific contacts with the TCR[24]. These residues are shown in Figure 3*a*, together with residues that determine Vβ specificity between enterotoxins SEA and SEE[2,41,42]. The SEA residues are in the C-terminal domain of the superantigen structure, in a loop between strand 9 and helix 5 of SEB. Mutation of the cysteine residues involved in the disulphide bond in SEA[43] and SEB[24] also prevents T-cell stimulation. All of these residues line a region between the two domains of the superantigen, defining a potential TCR-binding site that is located above and to one side of the MHC peptide-binding groove (Fig. 3*a*).

Implications for ternary complex with TCR

The hypervariable region 4 (HV4) of the Vβ domain of the TCR is important for superantigen interactions[14-18]. In a hypothetical model of the TCR[44], this region lies on an exposed face of the Vβ domain (shown in white in Fig. 3*b*). Direct binding studies with a soluble TCR β-chain[45] indicate that these interactions may be sufficient for formation of an MHC-superantigen-TCR complex.

The juxtaposition of the HV4 region of the TCR with the superantigen residues involved in TCR interactions leads to a model with interesting implications for the formation of the ternary complex between MHC, SEB and TCR (Fig. 3*b*). The complementarity-determining regions (CDRs) of the TCR are oriented over the MHC peptide-binding site, with the Vβ domain bound to SEB and the Vα domain above the class II β1 domain. This model is consistent with a role for both the TCR α-chain and MHC polymorphism in modulating super-

TABLE 1 Data collection and refinement statistics

Data	Cell dimensions (Å)	Resolution (Å)	R_{sym} (%)*	Completeness (%)
Crystal form I, space group $P2_12_12_1$				
Native	95.0 × 114.7 × 149.8	30.0–2.7	5.7	86
Synchrotron		2.8–2.7	32.1	87
Crystal form II, space group $P2_12_12_1$				
Native	95.8 × 127.0 × 183.8	30.0–4.35	11.4	82
GX-13		4.69–4.35	31.0	63

Refinement statistics						
Resolution (Å)	No. of reflections (working set)	No. of atoms†	R.m.s. bonds (Å)‡	R.m.s. angles‡	R_{cryst} (%)§	R_{free} (%)‖
6.0–2.7	31,557	9,400	0.017	2.16	25.7	32.7

A papain-solubilized form of the human class II MHC molecule, HLA-DR1 (DRA, DRB1*#0101*)[58] was co-crystallized with SEB in a 1:1 molar ratio (final total protein concentration, 15 mg ml^{-1}) from a stock solution in 10 mM Tris buffer, pH 7.5. Crystals were grown by vapour diffusion, by mixing 2 µl protein solution with 2 µl well solution containing 10 mM sodium acetate, pH 4.7, 10% ethylene glycol, and 12–20% PEG4000 (Fluka), at 25 °C. SEB was obtained as a lyophilized powder from Sigma, or Toxin Technology, from culture supernatants of an SEB-producing strain of *S. aureus* (Toxin Technology) and as a kind gift from M. Sax. Two crystal forms grew under the same conditions. Data for crystal form I were collected from crystals flash-frozen at −165 °C. Data for crystal form II were collected from 8 crystals at 25 °C and merged using the Buddha[59] and CCP4 programs[60]. Data were collected using a Nicolet/Xentronics area detector and Elliot GX-13 rotating-anode X-ray source with Franks double-mirror optics, and also at the Cornell High Energy Synchrotron Source (CHESS) F-1 beamline, using Kodak phosphor-image plates. CHESS data were processed with Denzo and Scalepack (Z. Otwinowski, personal communication). DR1–SEB SIR/anomalous phased electron density maps were iteratively averaged with DR1–SIR electron density maps and a model of the HLA-DR1 molecule was built as described[11]. The SEB region was not easily interpretable, so the DR1 model was improved, first by building into single DR1 domain omit maps, comparing maps calculated with both model and experimental SIR/anomalous phases, and maps calculated with DR1 model phases. Cycles of building and refinement of the DR1 model improved the SEB regions and a partial polyalanine model of SEB was built. This partial model of the DR1–SEB structure was used to solve the second, low-resolution DR1:SEB crystal form by molecular replacement using the Navazza suite of programs[61], giving an initial *R*-factor of 40.5% and a correlation coefficient of 48.4% from 8–4.3 Å. Iterative four-fold real-space averaging between the two space groups was carried out using the Bricogne package of programs[62], generating the electron density map shown in Fig. 1*a*. A 190-residue polyalanine trace of SEB was built and used to phase five higher-resolution (3.5 Å) maps, where 20% of the SEB polyalanine trace was omitted. Side chains were built into interpretable electron density, and the model was improved by further cycles of building, refinement and iterative two-fold non-crystallographic real-space averaging. The resolution was gradually extended from 3.5 to 2.7 Å. At all stages, refinement paths were taken that minimized the free *R*-factor[63]. As refinement proceeded, a number of loops in the SEB structure remained untraceable. These loops include residues 57–60 and 99–110 of the first domain and residues 122–126, 176–182, and the N- and C-terminal residues 1 and 236–239 of the second domain. In addition, three regions in the C-terminal domain of SEB do not show unambiguous side-chain density, including the N-terminal SEB region (residues 1–11) β-strands 6 and 7 (residues 127–154), and the C-terminal region of helix 4 and the adjacent loop residues (residues 169–175). In general, density for the second domain is less well defined. Refinement shows a dramatic difference in average atomic *B*-factor values between the HLA-DR1 molecule and the SEB molecule. The average Cα *B*-factor is 30 Å^2 for DR1, 55 Å^2 for the N-terminal domain of SEB1 (SEB1 is one of the SEB molecules in the asymmetric unit), and 70–80 Å^2 for the C-terminal domain, showing a radial increase from the DR1–SEB interface (Fig. 1). Given the high temperature factors for SEB, it was important to verify the SEB model. An independently refined model of HLA-DR1 (ref. 20), with no phase bias for the SEB model, was used to calculate $|2F_{obs} - F_{calc}|$ averaged and unaveraged omit maps. The omit maps verified the overall placement of the SEB molecule in the map, indicating a deteriorating map quality for SEB regions in the following order: N-terminal domain (SEB1) > C-terminal domain (SEB1) ~ N-terminal domain (SEB2) > C-terminal domain (SEB2), where SEB1 and SEB2 refer to the two SEB molecules in the asymmetric unit. In the final stages, the independently determined structure of the SEC3 molecule was compared to the SEB model, leading to rebuilding of difficult regions of the SEB, including three loops (29–32, 201–203 and 224–229), the N-terminal strand (residues 2–13), and the C-terminal residues of helix 4 and the adjacent loop (157–175). Fewer than 5% of the Φ/Ψ angles lie outside allowed regions of a Ramachandran plot.

* $R_{sym} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity, and $\langle I \rangle$ is the average intensity from several measurements.

† 9,400 non-hydrogen atoms represents 93% of the expected atoms for the DR1–SEB complex. Water molecules have not yet been included in this refinement.

‡ R.m.s. bond angles and lengths are r.m.s. deviations from ideal values.

§ *R*-factor calculated with working set reflections greater than 2σ.

‖ *R*-factor calculated with 3,383 reflections removed before automated refinement and greater than 2σ.

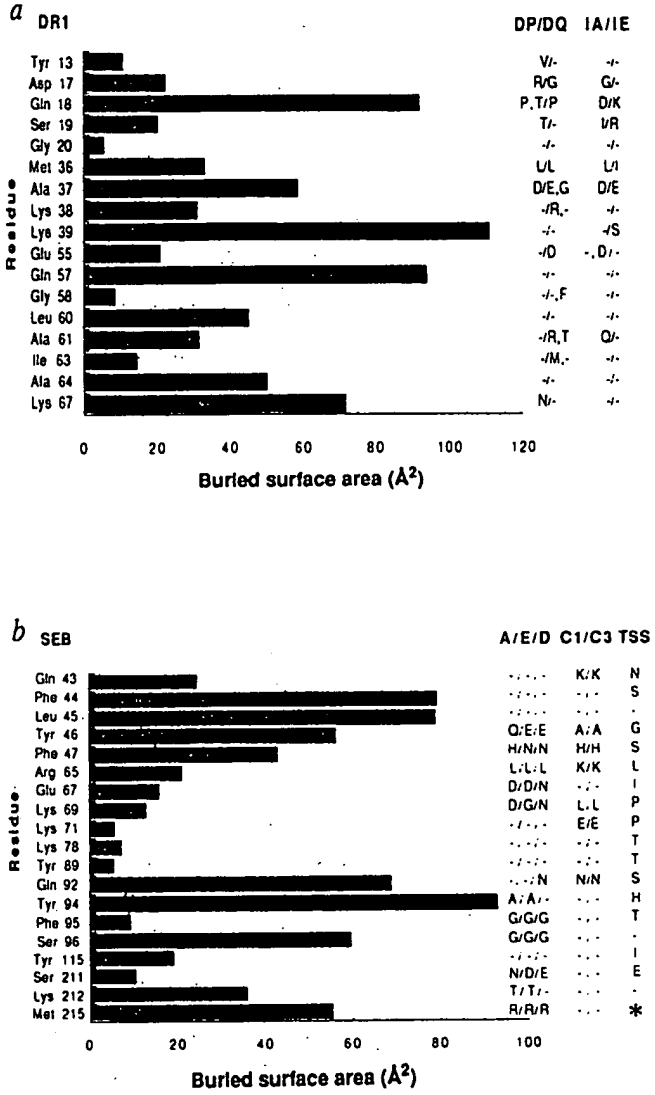


FIG. 5 Residues involved in the DR1–SEB interaction vary between class II isotypes and related superantigens. *a*, Plot of the buried solvent-accessible surface area[22] for each residue of the HLA-DR1 molecule involved in SEB binding. Corresponding residues in HLA-DP, HLA-DQ, mouse I–A, and I–E molecules is indicated to the right of the plot. *b*, Plot of the buried solvent-accessible surface area for each residue of the SEB molecule involved in HLA-DR1 binding. Corresponding residues in related superantigens are listed to the right for SEA, SED, SEE, SEC1, SEC3 and TSST-1. The TSST-1 residue alignment is based on the structural alignment of TSST-1 and SEB[38,39]. Surface areas were calculated with the program Access[22] as described in Fig. 4 legend. A dash is used for residues identical to DR1 or SEB and an asterisk indicates a deletion.

antigen stimulation through direct TCR–MHC interactions[16,24,26,29,46–53].

However, the DR1–SEB complex suggests that the interactions between TCR and MHC molecules during superantigen stimulation differ from the interactions involved in antigenic peptide stimulation. Residues of the DR1 α1-domain α-helix that are usually exposed and might interact with the TCR are partially or completely buried in the complex by SEB residues 92–96 (Fig. 3*c*). These MHC surface residues (at positions 55, 57, 58, 60, 61, 63, 64, 67) influence T-cell stimulation when mutated[12,13], indicating that this region of the DR α1-domain α-helix is important for TCR recognition of peptide antigens and arguing for an unconventional mode of interaction between TCR and MHC during superantigen stimulation.

Discussion

The crystal structure of the DR1–SEB complex shows that SEB binds to the α1 domain of class II molecules, positioning a TCR-binding site above and to the side of the MHC peptide-binding site. Antigenic peptides are not inhibitors of SEB stimulation[13] and the structure demonstrates that peptides and SEB occupy two distinct regions of the class II MHC molecule. In the DR1–SEB crystal, electron density is observed for 13 residues of an extended peptide chain, corresponding to a mixture of self peptides bound to DR1 (T.S.J., manuscript in preparation). The details of the DR1–SEB interaction indicate how superantigen affinity could be modulated for different class II isotypes.

The interaction of related bacterial superantigens with class II molecules may differ from that seen in the DR1–SEB structure. Two lines of evidence support such a view. The first is that different superantigens do not all cross-compete in binding studies. SEB and TSST-1 do not competitively inhibit each other or completely block SEA binding to HLA-DR[28,30,31], indicating the existence of independent binding sites. However, SEA is able to compete effectively with both SEB and TSST-1 for binding[31], suggesting that these sites may overlap. In addition, mutational studies of HLA-DR and SEA suggest that SEA has a different binding site. Histidine 81 of the class II β-chain[32,33] is important for SEA binding to DR, but has no effect on SEB or TSST-1 binding. This interaction may be mediated by a zinc atom bound to a metal coordination site found in SEA, SED and SEE[54]. SEB does not require zinc to bind class II molecules, and the residues in SEA that bind zinc are in the C-terminal domain, far from the DR1–SEB interface, indicating that SEA has a different mode of binding from SEB.

Important features of the DR1–SEB interaction are conserved in SEA, suggesting that SEA may have evolved two distinct modes of binding to class II molecules, one similar to SEB and another zinc-mediated interaction with the class II β-chain. This is consistent with the available mutational and binding data.

Binding of one SEA molecule to both α-chain and β-chain sites on one class II molecule is unlikely, considering the DR1–SEB structure, the competition data, and the zinc-binding site of SEA. However, one SEA molecule could potentially crosslink two class II molecules, with the N-terminal domain interacting with the α1 domain of class II (as observed for SEB) and the C-terminal domain interacting with the β-chain of another class II molecule. This type of crosslinking might explain the ability of these superantigens to stimulate T cells at concentrations well below their measured K_d values.

The crystal structure also supports a model of ternary complex formation in which the MHC interaction with the TCR is distinct from the peptide-mediated interaction. A stretch of residues located in the SEB disulphide loop lies across the α1 α-helix of DR1, covering residues that have been implicated in TCR recognition of peptide–MHC complexes. This suggests that superantigens may not take advantage of any residual affinity of TCR for MHC derived from positive selection during thymic education. However, the location of SEB residues that interact with TCR and of TCR residues that interact with superantigens suggests that the TCR may still be positioned in close proximity to the class II peptide-binding site. This may explain how TCR α-chains and MHC polymorphisms can modulate superantigen stimulation. Superantigens may have evolved to bind class II MHC molecules, not in order to use existing MHC–TCR interactions to stimulate T cells, but rather to take advantage of additional signals and organization inherent in conventional antigen presentation.

Superantigens have been directly implicated in a number of diseases and it has been suggested that they might be involved in the generation of autoimmunity[55] by stimulating existing autoreactive T cells. It has been shown that SEB can induce relapsing paralysis in mice that have previously been immunized with a peptide that induces experimental autoimmune encephalomyelitis[56,57]. This indicates that the powerful T-cell-activation properties of superantigens may be important in the development and relapse of autoimmune disorders. An understanding of the structural requirements for the action of these superantigens may further the development of strategies to control the onset and progression of disease. □

Received 14 February; accepted 31 March 1994.

1. Herman, A., Kappler, J. W., Marrack, P. & Pullen, A. M. *A. Rev. Immunol.* **9,** 745–772 (1991).
2. Irwin, M. J., Hudson, K. R., Ames, K. T., Fraser, J. D. & Gascoigne, N. R. J. *Immun. Rev.* **131,** 61–78 (1993).
3. Micusan, V. V. & Thibodeau, *J. Semin. Immun.* **5,** 3–11 (1993).
4. Marrack, P. *et al. Immun. Rev.* **131,** 79–92 (1993).
5. Marrack, P., Blackman, M., Kushnir, E. & Kappler, J. *J. exp. Med.* **171,** 455–464 (1990).
6. Miethke, T. *et al. J. exp. Med.* **175,** 91–98 (1992).
7. Kotzin, B. L., Leung, D. M., Kappler, J. & Marrack, P. *Adv. Immun.* **54,** 99–166 (1993).
8. Germain, R. M. & Margulies, D. H. *A. Rev. Immun.* **11,** 403–450 (1993).
9. Madden, D. R., Garboczi, D. N. & Wiley, D. C. *Cell* **75,** 693–708 (1993).
10. Matsumura, M., Fremont, D. H., Peterson, P. A. & Wilson, I. A. *Science* **257,** 927–934 (1992).
11. Brown, J. H. *et al. Nature* **364,** 33–39 (1993).
12. Jorgensen, J. L., Reay, P. A., Ehrich, E. W. & Davis, M. M. *A. Rev. Immun.* **10,** 835–873 (1992).
13. Dellabona, P. *et al. Cell* **62,** 1115–1121 (1990).
14. Choi, Y. *et al. Nature* **346,** 471–473 (1990).
15. Cazenave, P. A. *et al. Cell* **63,** 717–728 (1990).
16. Pullen, A. M., Wade, T., Marrack, P. & Kappler, J. W. *Cell* **61,** 1365–1374 (1990).
17. Pullen, A. M., Bill, J., Kubo, R. T., Marrack, P. & Kappler, J. W. *J. exp. Med.* **173,** 1183–1192 (1991).
18. White, J., Pullen, A., Choi, K., Marrack, P. & Kappler, J. W. *J. exp. Med.* **177,** 119–125 (1993).
19. Swaminathan, S., Furey, W., Pletcher, J. & Sax, M. *Nature* **359,** 801–806 (1992).
20. Stern, L. J. *et al. Nature* **368,** 215–221 (1994).
21. Buelow, R. *et al. J. Immun.* **148,** 1–6 (1992).
22. Lee, B. & Richards, F. M. *J. molec. Biol.* **55,** 379–400 (1971).
23. Connolly, M. L. *J. appl. Crystallogr.* **16,** 548–558 (1983).
24. Kappler, J. W., Herman, A., Clements, J. & Marrack, P. *J. exp. Med.* **175,** 387–396 (1992).
25. Scholl, P. R. *et al. J. Immun.* **144,** 226–230 (1990).
26. Herman, A., Croteau, G., Sekaly, R. P., Kappler, J. & Marrack, P. *J. exp. Med.* **172,** 709–717 (1990).
27. Herrmann, T., Accolla, R. S. & MacDonald, H. R. *Eur. J. Immun.* **19,** 2171–2174 (1989).
28. Fraser, J. D. *Nature* **339,** 221–223 (1989).
29. Mollick, J. A., Chintagumpala, M., Cook, R. G. & Rich, R. R. *J. Immun.* **146,** 463–468 (1991).
30. Scholl, P. R., Diez, A. & Geha, R. S. *J. Immun.* **143,** 2583–2588 (1989).
31. Chintagumpala, M. M., Mollick, J. A. & Rich, R. R. *J. Immun.* **147,** 3876–3881 (1991).
32. Herman, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **88,** 9954–9958 (1991).
33. Karp, D. R. & Long, E. O. *J. exp. Med.* **175,** 415–424 (1992).
34. Karp, D. R., Teletski, C. L., Scholl, P., Geha, R. & Long, E. O. *Nature* **346,** 474–476 (1990).
35. Panina-Bordignon, P., Fu, X.-t., Lanzavecchia, A. & Karr, R. W. *J. exp. Med.* **176,** 1779–1784 (1992).
36. Braunstein, N. S., Weber, D. A., Wang, X. C., Long, E. O. & Karp, D. *J. exp. Med.* **175,** 1301–1305 (1992).
37. Harris, T. O. *et al. Infect. Immun.* **61,** 3175–3183 (1993).
38. Prasad, G. S. *et al. Biochemistry* **32,** 13761–13766 (1993).
39. Acharya, K. R. *et al. Nature* **367,** 94–97 (1994).
40. Thibodeau, J., Labreque, N., Denis, F., Huber, B. & Sékaly, R.-P. *J. exp. Med.* **179,** 1029–1034 (1994).
41. Hudson, K. R., Robinson, H. & Fraser, J. D. *J. exp. Med.* **177,** 175–184 (1993).
42. Mollick, J. A., McMasters, R. L., Grossman, D. & Rich, R. R. *J. exp. Med.* **177,** 283–293 (1993).
43. Grossman, D., Van, M., Mollick, J. A., Highlander, S. K. & Rich, R. R. *J. Immun.* **147,** 3274–3281 (1991).
44. Chothia, C., Boswell, D. R. & Lesk, A. M. *EMBO J.* **7,** 3745–3755 (1988).
45. Gascoigne, N. R. J. & Ames, K. T. *Proc. natn. Acad. Sci. U.S.A.* **88,** 613–616 (1991).
46. Yagi, J., Rath, S. & Janeway, C. A. Jr *J. Immun.* **147,** 1398–1405 (1991).
47. Herrmann, T., Maryanski, J. L., Romero, P., Fleischer, B. & MacDonald, H. R. *J. Immun.* **144,** 1181–1186 (1990).
48. Smith, H. P., Le, P., Woodland, D. L. & Blackman, M. A. *J. Immun.* **149,** 887–896 (1992).
49. Vacchio, M. S., Kanagawa, O., Tomonari, K. & Hodes, R. J. *J. exp. Med.* **175,** 1405–1408 (1992).
50. Blackman, M. A., Smith, H. P., Le, P. & Woodland, D. L. *J. Immun.* **151,** 556–565 (1993).
51. Gascoigne, N. R. J. *Semin. Immun.* **5,** 13–21 (1993).
52. Waanders, G. A., Lussowe, A. R. & MacDonald, H. R. *Int. Immun.* **5,** 55–61 (1993).
53. Woodland, D. L. *et al. J. exp. Med.* **177,** 433–442 (1993).
54. Fraser, J. D. & Hudson, K. R. *Res. Immun.* **144,** 188–193 (1993).
55. Pallard, X. *et al. Science* **253,** 325–329 (1991).
56. Brocke, S. *et al. Nature* **365,** 642–644 (1993).
57. Soos, J., Schiffenbacher, J., Wegrzyn, L. & Johnson, H. M. *J. Immun.* **150,** 192A (1993).
58. Gorga, J. C., Horejsi, V., Johnson, D. R., Raghupathy, R. & Strominger, J. L. *J. biol. Chem.* **262,** 16087–16094 (1987).
59. Blum, M., Metcalf, P., Harrison, S. C. & Wiley, D. C. *J. appl. Crystallogr.* **20,** 235–242 (1987).
60. *CCP4: A Suite of Programs for Protein Crystallography* (SERC Collaborative Computing Project No. 4, Daresbury Laboratory, Warrington, UK, 1979).
61. Navaza, J. *Acta crystallogr.* **A43,** 645–653 (1987).
62. Bricogne, G. *Acta crystallogr.* **A32,** 832–847 (1976).
63. Brünger, A. T. *Nature* **355,** 472–475 (1992).
64. Jones, T. A., Zou, J.-Y., Cowan, S. W. & Kjeigaard, M. *Acta crystallogr.* **A47,** 110–119 (1991).
65. Kraulis, P. J. *J. appl. Crystallogr.* **24,** 945–949 (1991).

ACKNOWLEDGEMENTS. We thank P. Rosenthal, S. Garman, M. Elsen and staff of the Cornell High Energy Synchrotron Source MacCHESS for help with data collection, P. Klimotovsky and A. Haykov for additional technical assistance; and M. Blum and other members of our groups for discussion. T.S.J. was supported by the Cancer Research Institute; J.H.B. by the Howard Hughes Medical Institute (HHMI); J.C.G. by the Juvenile Diabetes Foundation International; L.J.S. by a Damyon Runyon–Walter Winchell Cancer Fellowship and HHMI; R.G.U. by the Irvington Institute; Y.I.C. and C.U.S. by the Lucille P. Markey Foundation Trust; and J.L.S. by the NIH. D.C.W. is an investigator with the Howard Hughes Medical Institute. Coordinates will be deposited in the Brookhaven Protein Databank and are available from the authors.

Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9287–9291, October 1991
Immunology

Monoclonal antibody-targeted superantigens: A different class of anti-tumor agents

(staphylococcal enterotoxins/cytotoxic T cells/colon cancer cells)

MIKAEL DOHLSTEN*†, GUNNAR HEDLUND*†, EVA ÅKERBLOM*, PETER A. LANDO*, AND TERJE KALLAND*†‡

*Kabi Pharmacia Therapeutics AB, S-223 63 Lund, Sweden; and †Department of Tumor Immunology, The Wallenberg Laboratory, University of Lund, Lund, Sweden

Communicated by Hans Müller-Eberhard, June 27, 1991

ABSTRACT **The bacterial superantigen staphylococcal enterotoxin (SE) A (SEA) directs cytotoxic T lymphocytes (CTLs) expressing particular sequences of the T-cell receptor (TCR) β chain to lyse tumor cells expressing major histocompatibility complex (MHC) class II molecules, which serve as receptors for SEs. We now report that chemical conjugates of SEA and the colon carcinoma-reactive monoclonal antibodies (mAbs) C215 or C242 mediate T cell-dependent destruction of colon carcinoma cells lacking MHC class II molecules. SEA was covalently linked to the mAbs C215 and C242 via a PEG-based hydrophilic spacer. The C215-SEA conjugate targeted $CD4^+$ as well as $CD8^+$ CTLs to lyse a panel of colon carcinoma cells lacking MHC class II molecules. T-cell recognition of mAb-SEA conjugates was SEA specific, since SEB-selective T-cell lines with potent cytotoxic activity towards Raji cells coated with SEB did not respond to the C215-SEA conjugate. Unconjugated SEA did not induce T-cell lysis of MHC class II^- colon carcinoma cells but efficiently directed CTLs against MHC class II^+ Raji cells and certain interferon-treated MHC class II^+ colon carcinoma cells. These results suggest that SEA-mAb conjugates retain the SEA-related selectivity for certain TCR β-chain variable region (V_β) sequences but, in contrast to unconjugated SEA, mediate the TCR interaction in a MHC class II-independent manner. The cytotoxic activity mediated by C215-SEA and C242-SEA conjugates was blocked by excess of C215 mAb and C242 mAb, respectively, showing that the specificity in the targeting of mAb-SEA conjugates is defined by the antigen reactivity of the mAb. These results demonstrate that bacterial superantigens may be successfully conjugated to mAb with preserved T cell-activating capacity. The circumvention of MHC class II binding of SEs by conjugation to mAb suggests that such conjugates may find general application as antitumor agents, taking advantage of the extreme T cell-activating potency of superantigens.**

The collection of superantigens consists of bacterial exoproteins, such as the structurally related staphylococcal and streptococcal exotoxins, but also endogenous self superantigens, including the mammary tumor virus-encoded Mls antigens (1–3). They are characterized by the capacity to stimulate in a major histocompatibility complex (MHC) class II-dependent manner a high frequency of T cells bearing particular T-cell receptor (TCR) β-chain variable segments (V_β) (1, 4–10). Studies on the staphylococcal enterotoxins (SEs) A and B (SEA and SEB) and toxic shock syndrome toxin 1 have shown high-affinity binding to MHC class II molecules (4–6, 10–12). SEA, SEB, and toxic shock syndrome toxin 1 bind to a variety of different MHC class II isotypes and allotypes, and the binding seems to involve conserved peptide sequences expressed on both the MHC class II α and β chains (9–13). T cells recognizing the MHC class II–SE complex are activated to proliferation, cytokine production, and cytotoxicity (14–19). The SE-dependent cell-mediated cytotoxicity (SDCC) results in elimination of MHC class II-expressing SE-presenting target cells (17–19). Studies on fresh MHC class II^+ leukemic cells have suggested that the SDCC mechanisms may be a useful tool for therapeutic elimination of MHC class II^+ tumor cells (16). However, since MHC class II-expressing tumor types only represent a minority of the most frequent human tumors and systemic T-cell activation is expected to result in severe toxicity, it seems reasonable to assume that the SDCC mechanism will not have general application in the treatment of human malignant diseases. During the last decade monoclonal antibodies (mAbs) have been evaluated for tumor therapy, either as native antibodies or conjugated to radioactive isotopes, cytotoxic drugs, or plant toxins (20–23). Recent attempts have also included polyclonal activation of T lymphocytes by antibody heteroconjugates simultaneously recognizing tumor cells and the CD3/TCR complex on T cells (24–26). The direct binding of antibody heteroconjugates to the T cell may be a major drawback *in vivo*, counteracting mAb localization at the tumor site. In this report we demonstrate that conjugates between SEA and mAbs recognizing human colon cancer enable T cells to lyse colon carcinoma cells *in vitro*. The mAb-SEA conjugates direct cytotoxic T lymphocytes (CTLs) against target cells expressing the mAb-defined cell-surface antigen independent of their MHC expression. In contrast to antibody heteroconjugates, the mAb-SEA conjugates do not engage T cells prior to binding to the target cell. We believe that mAb–superantigen-based conjugates represent a novel and powerful approach to tumor therapy, which may have significant advantages in comparison with earlier described antibody-based therapies.

MATERIALS AND METHODS

Reagents. SEB was purchased from Toxin Technology (Madison, WI). Recombinant SEA was expressed in *Escherichia coli* and purified to homogeneity as described elsewhere (ref. 27). The following mAbs were used to detect monomorphic determinants on human MHC class II: HLA-DR, L243 and D1-12; HLA-DP, B7/21; and HLA-DQ, SK10 (HLA-Qw1 and -Qw3), BT3/4 (HLA-DQw1), and SFR16-P1.2 (HLA-DQw2 and -DQw3). The sources of these mAbs have been reported recently (27). The mAbs C215 (IgG2a) and C242 (IgG1) reacting with human colon cancer were obtained from L. Lindholm, Pharmacia Canag (Göteborg, Sweden). Rabbit anti-SEA serum was obtained from Phar-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SE, staphylococcal enterotoxin; SEA and SEB, SEs A and B; mAb, monoclonal antibody; TCR, T-cell receptor; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; V_β, β-chain variable regions; SDCC, SE-dependent cell-mediated cytotoxicity; FITC, fluorescein isothiocyanate.

‡To whom reprint requests should be addressed at * address.

macia. Fluorescein isothiocyanate (FITC)-labeled swine anti-rabbit and rabbit anti-mouse immunoglobulins were purchased from Dakopatts (Glostrup, Denmark).

Preparation of mAb-SEA Conjugates. Recombinant SEA was coupled to the C215 mAb or C242 mAb by the use of *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Pharmacia) and a 24-atom-long PEG-based hydrophilic spacer (*N*-hydroxysuccinimide ester of 17-iodoacetylamino-3,6,9,12,15-pentaoxaheptadecanoic acid) as recently described (ref. 28). Briefly, the ε-amino groups of the lysines in the mAb were randomly substituted with the PEG-based spacer, which resulted in 7–18 coupled spacers per mAb. One or two mercapto groups were substituted on the ε-amino groups of lysines in SEA by reaction with the SPDP reagent (29). The spacers ended with reactive iodine groups which reacted with the mercapto groups introduced on SEA, resulting in the formation of stable thioether linkages. The synthesized mAb-SEA conjugate was fractionated on a Superdex 200 HR 16/50 column (Pharmacia) and was eluted with 2 mM phosphate buffer, pH 7.5/0.15 M NaCl. Fractions with the desired product were pooled and analyzed by SDS/PAGE on Phast-Gel5 gradient 4-15 and silver staining (Pharmacia). The conjugates contained zero to three SEA molecules per mAb molecule (average one for C215-SEA and two for C242-SEA).

^{125}I-Labeled SEA (^{125}I-SEA) and C215-^{125}I-SEA Binding Assays. SEA or C215-SEA (5–10 μg) was mixed with 0.5–1.0 mCi (1 Ci = 37 GBq) of ^{125}I (carrier-free NaI, 105 mCi/ml; DuPont/NEN) in 200 μl of phosphate-buffered saline (PBS). One Iodo-Bead (Pierce) was added to the mixture, and after 15 min of incubation at room temperature, 2-mercaptoethanol was added to stop the reaction. Iodinated proteins were separated from free iodine by gel filtration (PD-10 Sephadex G-25M, Pharmacia). When evaluating the inhibition of ^{125}I-SEA and C215-^{125}I-SEA to Raji cells by SEA and C215-SEA, respectively, 10^6 Raji cells in 50 μl of PBS with 1% bovine serum albumin were incubated with various concentrations of unlabeled inhibitor for 30 min at room temperature. Fifty microliters of ^{125}I-SEA and 50 μl of C215-^{125}I-SEA were then added, and the mixture was further incubated for 15 min. Cell-associated radioactivity was separated from free ^{125}I-SEA and C215-^{125}I-SEA by centrifugation of the Raji cells on a 40% Ficoll-Paque (Pharmacia) cushion.

Cell Lines. The B-cell lymphoma line Raji and the colon carcinoma lines SW620, COLO 205, and WiDr were obtained from American Type Culture Collection and cultured in R-medium [RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 1 mM nonessential amino acids, 50 μM 2-mercaptoethanol, and 1 mM pyruvate (Flow Laboratories)]. T-cell lines were established by stimulation of human peripheral blood lymphocytes with SEA or SEB (1 ng/ml) as detailed earlier (15, 16). These T-cell lines were all >99% $CD3^+$. $CD4^+$ and $CD8^+$ sublines were established after separation by positive selection with magnetic beads precoated with anti-CD4 or anti-CD8 mAb (Dynabeads M-450, Dynal A/S, Oslo, Norway). These sublines were >92% pure with respect to CD4 or CD8.

Cytotoxicity Assay. Cytotoxicity was measured at various effector/target cell ratios in a standard 4-hr ^{51}Cr-release assay as described (15). SEs or conjugates were added at various concentrations directly into the assay or were used for preincubation of target cells. Preincubation was performed at 37°C for 30 min followed by extensive washing of the cells.

Analysis by Flow Cytometry. Flow cytometric analyses were performed with indirect immunofluorescence and with standard settings on a FACStarPLUS flow cytometer (Becton Dickinson).

RESULTS

Binding Characteristics of the C215-SEA Conjugate. Binding of mAb C215 and the C215-SEA conjugate to COLO 205 cells, which express the C215 antigen but not MHC class II molecules, was analyzed by flow cytometry with FITC-labeled anti-mouse IgG antibodies. The binding of the conjugate to COLO 205 cells was similar to that of the parental mAb (Fig. 1*A*). Extensive titrations of unconjugated mAb and C215-SEA

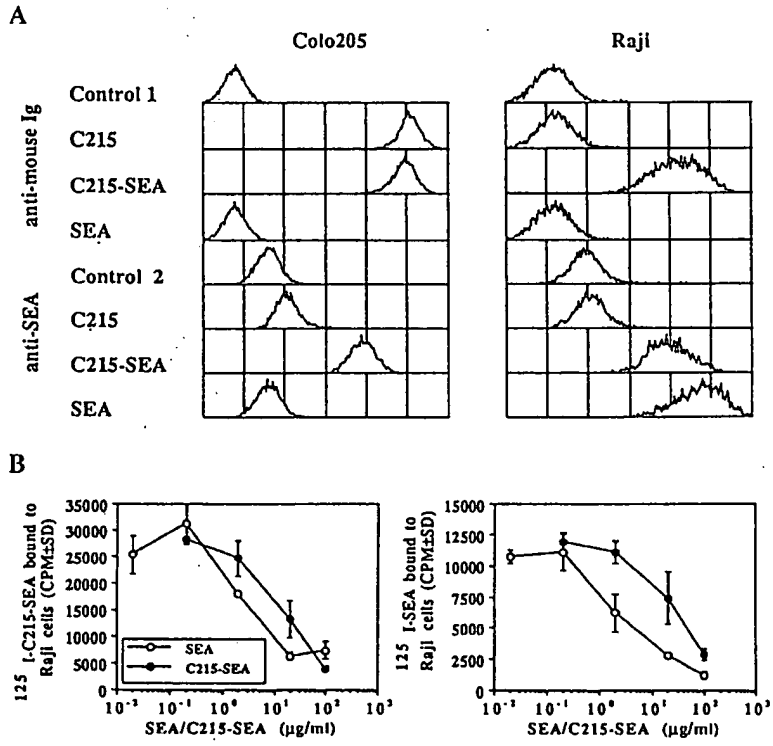


FIG. 1. Binding of C215-SEA, C215 mAb, and SEA to COLO 205 and Raji cells. (*A*) Fluorescence-activated cell sorter analysis on binding of C215-SEA conjugate, C215 mAb, and SEA to COLO 205 and Raji cells was performed by the use of FITC-labeled rabbit anti-mouse immunoglobulin (control 1) or rabbit anti-SEA sera/FITC-labeled swine anti-rabbit immunoglobulin (control 2). (*B*) Inhibition of ^{125}I-SEA and C215-^{125}I-SEA binding to Raji cells by unlabeled SEA and C215-SEA. The inhibitors were added 30 min before the labeled reagent.

conjugate demonstrated similar binding characteristics, with saturated binding detected at about 3 μg/ml (data not shown). The C215-SEA conjugate, but not unconjugated SEA or C215 mAb, was detected on COLO 205 cells as analyzed by the use of rabbit anti-SEA antibodies and FITC-labeled swine anti-rabbit immunoglobulin (Fig. 1*A*). We demonstrated earlier (4) that SEA bound with high affinity to MHC class II molecules on Raji cells. Fluorescence-activated cell sorter analysis showed that SEA and C215-SEA, but not unconjugated C215, bound strongly to Raji cells (Fig. 1*A*). The binding of C215-SEA to Raji cells was efficiently blocked by SEA but not by C215 (data not shown). Thus, in addition to mAb specificity, the C215-SEA conjugate has retained the capacity of SEA to bind to cells expressing MHC class II molecules. To compare the relative MHC class II binding affinity of SEA and C215-SEA, respectively, we utilized a cell-binding assay with ^{125}I-SEA and C215-^{125}I-SEA. Inhibition studies showed that ^{125}I-SEA was displaced in a dose-dependent manner by SEA and C215-SEA mAb (Fig. 1*B*). Similarly, C215-^{125}I-SEA binding to Raji cells was efficiently blocked by SEA and C215-SEA, which indicates that the C215-SEA binding is specific for the MHC class II molecule (Fig. 1*B*). Assuming that the C215-SEA conjugate contains about 15% SEA, the conjugate apparently displays identical MHC class II binding as SEA on a molar basis.

T-Cell Targeting by the C215-SEA Conjugate. The C215-SEA conjugate efficiently directed SEA-responsive CTLs to mediate cytotoxicity against the SW620 colon carcinoma cells, whereas a mixture of unconjugated SEA and C215 mAb had no effect (Fig. 2). Cytotoxicity against SW620 cells was induced by C215-SEA at effector-to-target ratios as low as 3:1, while unconjugated SEA and C215 mAb lacked effect even at 30:1. Lysis was recorded at 3 μg of the conjugate per ml, whereas SEA at 1000-fold higher concentrations only had marginal effects (Fig. 2). The SW620 cell line did not constitutively or after interferon treatment express surface MHC class II molecules, as analyzed by immunoprecipitation and flow cytometry with a panel of mAbs against the HLA-DR, HLA-DP, and HLA-DQ isotypes (27). Furthermore, Northern blot analysis demonstrated absence of HLA-DRα, HLA-DRβ, invariant chain, and HLA-DZα transcripts in SW620 cells (27). C215-SEA conjugate-induced cytotoxicity was observed against several MHC class II$^-$ C215$^+$ colon carcinoma cell lines, including WiDr, COLO 205, and SW620 (Fig. 2). Unconjugated SEA (Fig. 2) and C215 mAb (data not shown) demonstrated only marginal effect on MHC class II$^-$ colon carcinoma cells lines, while SEA induced CTL targeting against MHC class II$^+$ Raji cells (Fig. 2). CTL targeting against Raji cells was efficiently induced by SEA at 0.003 ng/ml, whereas C215-SEA conjugate at 30 ng/ml was required to induce a comparable half-maximal cell lysis, indicating an extremely low efficiency of the C215-SEA conjugate against MHC class II$^+$ C215$^-$ cells compared with SEA.

To analyze the influence of concomitant expression of C215 and MHC class II molecules on a target cell in comparison with separate expression of either of these molecules, we used interferon treatment of COLO 205 cells to induce surface MHC class II expression. Dose–response analysis with untreated MHC class II$^-$ COLO 205 cells showed

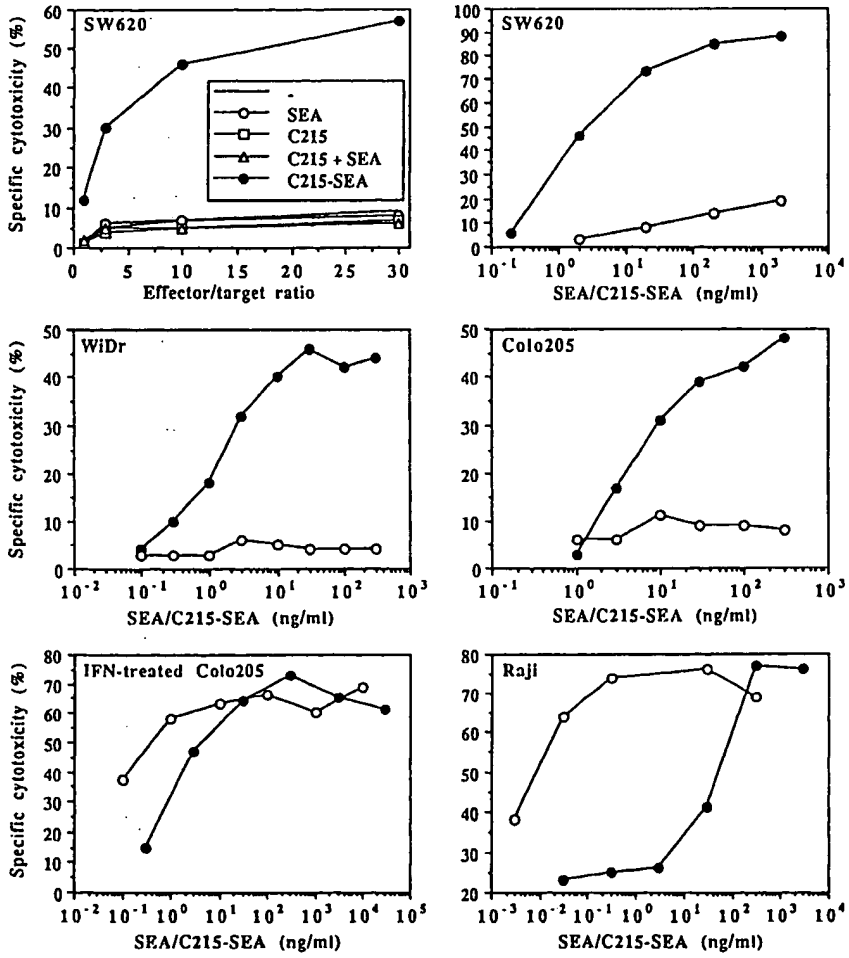


FIG. 2. C215-SEA directs CTLs against MHC class II$^-$ colon carcinoma cells. (*Top Left*) Effect of SEA-responsive CTLs against SW620 cells at various effector-to-target ratios in the absence (−) or presence of C215-SEA, SEA, C215, and a mixture of C215 and SEA (C215+SEA) at a concentration of 1 μg/ml of each additive. Other panels show the capacity of C215-SEA and SEA to target SEA-responsive CTLs against the C215$^+$ MHC class II$^-$ colon carcinoma cell lines SW620, COLO 205, and WiDr; MHC class II$^+$ C215$^+$ interferon-treated COLO 205 cells; and C215$^-$ MHC class II$^+$ Raji cells. Effector-to-target ratio was 30:1. Addition of unconjugated C215 mAb at several concentrations did not induce any CTL targeting against these cell lines. Fluorescence-activated cell sorter analysis on SW620 cells, COLO 205, and WiDr cells using mAbs against HLA-DR, -DP, and -DQ failed to detect any surface MHC class II expression, whereas abundant expression of HLA-DR, -DP, and -DQ was detected on Raji cells and HLA-DR and -DP were detected on interferon-treated COLO 205 cells. COLO 205 cells were treated with 1000 units of recombinant γ interferon per ml for 48 hr prior to use in the CTL assay.

Table 1. $CD4^+$ and $CD8^+$ CTLs lyse colon carcinoma cells presenting the C215-SEA conjugate

Effector*	Target	% cytotoxicity		
		Control	SEA	C215-SEA
$CD4^+$	SW620	2	5	50
$CD4^+$	Raji	0	41	43
$CD8^+$	SW620	0	1	23
$CD8^+$	Raji	2	72	68

*The CTLs (SEA-3) were used at effector-to-target ratio of 30:1 in the absence (control) or presence of SEA and C215-SEA at 1 μg/ml.

sensitivity to lysis at low concentrations of the C215-SEA conjugate but resistance to unconjugated SEA and C215 mAb (Fig. 2). Interferon γ treatment of COLO 205 cells resulted in strong expression of HLA-DR and HLA-DP molecules (27) and sensitivity to CTL lysis at similar concentrations of SEA and C215-SEA (Fig. 2).

C215-SEA Conjugate Targets $CD4^+$ and $CD8^+$ SEA-Responsive CTLs but Not SEB-Responsive CTLs. Both $CD4^+$ and $CD8^+$ CTLs mediated conjugate-dependent cytotoxicity against human colon carcinoma cells (Table 1). Unconjugated SEA failed to induce lysis of SW620 cells but targeted $CD4^+$ and $CD8^+$ CTL against MHC class II^+ Raji cells (Table 1). C215-SEA conjugate efficiently targeted SEA-responsive CTLs against SW620 and Raji cells but failed to target SEB-responsive CTLs (Fig. 3). In contrast, the SEB-responsive CTLs demonstrated strong cytotoxicity against SEB-coated Raji cells (Fig. 3).

Specificity of the mAb-SEA Conjugate Is Defined by the mAb. To demonstrate that the target selectivity of the mAb-SEA conjugate is entirely dependent on the mAb specificity, we performed criss-cross inhibition experiments with unconjugated C215 and C242 mAbs and C215-SEA and C242-SEA conjugates. Cytotoxicity mediated by C215-SEA and C242-SEA was blocked by addition of excess unconjugated C215

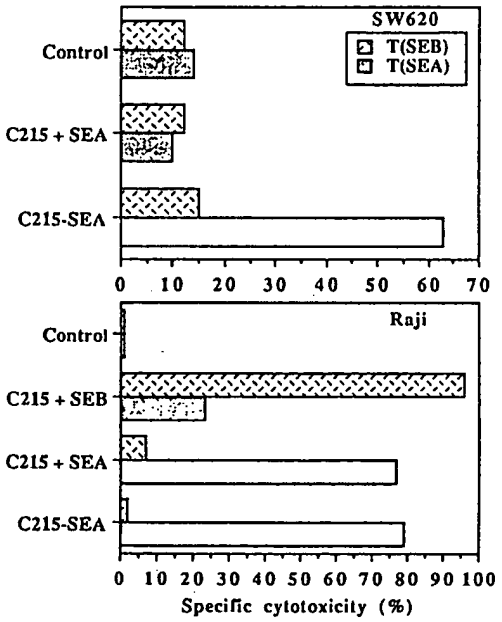


FIG. 3. Lysis of C215-SEA-coated colon carcinoma cells is mediated by SEA but not SEB-responding CTLs. Autologous SEA- and SEB-selective T-cell lines were used at an effector-to-target ratio of 10:1 against SW620 and Raji target cells in the absence (control) or presence of C215-SEA conjugate, a mixture of unconjugated C215 mAb and SEA (C215+SEA), or unconjugated C215 mAb and SEB (C215+SEB) at a concentration of 1 μg/ml of each additive.

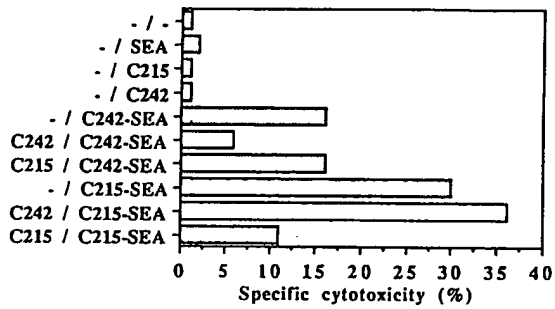


FIG. 4. C215-SEA- and C242-SEA-induced CTL targeting against colon carcinoma cells depends on the antigen selectivity of the mAb. Lysis of COLO 205 cells by a SEA-responsive CTL line in the presence of C215-SEA and C242-SEA conjugates (3 μg/ml) is blocked by addition of unconjugated C215 and C242 mAbs (30 μg/ml), respectively. The unconjugated mAbs or control medium (−) were added to the target cells 10 min prior to addition of the conjugates.

and C242, respectively but was not influenced by addition of the irrelevant mAb binding to the same target cell (Fig. 4) or high concentrations of isotype-specific control antibodies (data not shown).

DISCUSSION

CTLs with specificity for antigens expressed on tumor cells have been demonstrated in patients with malignant melanoma and renal cancer (30, 31). However, they are in most cases infrequent and obviously not capable of protecting the host against the growing tumor. The superantigen SEA allows activation of a frequency of T cells (>10%) even higher than that observed during the response to allogenic MHC in organ transplantation. Since the latter inevitably results in rejection of the transplant, utilization of SEA to direct a high frequency of T cells towards a tumor may hopefully ensure a similar outcome. In this paper we describe such an approach to tumor therapy. Conjugation of SEA to mAb directed against human colon carcinomas provided an agent that was able to selectively target SEA-responsive CTLs against the tumor cells. Earlier studies in our and other laboratories have demonstrated that binding of SEA to MHC class II molecules is a prerequisite for subsequent activation and targeting of T cells (4–6, 15). The SEA-mAb-mediated cytotoxicity apparently is MHC class II independent and does not require antigen-specific effector CTLs. The specificity at the target level is defined by the mAb and at the CTL level by the expression of relevant TCR V_β sequences, suitable for interaction with SEA.

The SW620 colon carcinoma cell line, which lacked mRNA transcripts for HLA-DRα, HLA-DRβ, HLA-DZα, and invariant chain and failed to express surface MHC class II antigens as analyzed by either immunoprecipitation or flow cytometry (27) efficiently presented C215-SEA but not SEA to CTLs. Conjugate-dependent killing not only was restricted to SW620 cells but also was recorded for MHC class II^- COLO 205 and WiDr cell lines as well as freshly isolated MHC class II^- human colon carcinoma cells (data not shown). The existence of MHC class II-independent T-cell activation induced by bacterial superantigens is supported by recent studies by Fleischer and co-workers (32). They demonstrated that SEB bound to silica beads activated T cells, provided that CD8- or CD2-mediated costimulatory signals were delivered. It is reasonable to suggest that soluble SEA interacts with insufficient low affinity to TCR V_β to activate T cells, but when presented in multivalent form on a cell surface or bead, an interaction with enhanced avidity is provided. Delivering costimulatory signals through the adhesion structures

LFA-1/ICAM-1, CD2/LFA-3, or CD8 may play an important role in exceeding the activation threshold (19, 32).

In comparison with unconjugated SEA, the C215-SEA conjugate retains similar MHC class II binding ability but has about 0.1% activity in MHC class II-dependent CTL targeting. This may be interpreted as a possible sterical hindrance by the conjugated C215 mAb when SEA is bound to MHC class II molecules, whereas binding to $C215^+$ on a cell surface by the mAb allows an efficient interaction between SEA and the TCR on the effector cell. In a therapeutic situation, presentation of the conjugate on normal MHC class II-expressing cells such as monocytes, B cells, and activated T cells is undesirable. Binding to normal cells would prevent the conjugate from reaching the tumor, and normal MHC class II^+ cells have been shown to be sensitive to SDCC (16). Although the present conjugation procedure apparently has markedly reduced the SDCC function of the C215-SEA conjugate, it would be of importance to further perturb MHC class II-dependent CTL activity by reducing the binding affinity of the C215-SEA conjugate for MHC class II molecules. We have recently demonstrated that a recombinant C-terminal fragment of SEA contains MHC class II binding determinants (G.H., unpublished data). Similarly, studies on SEC1 and toxic shock syndrome toxin 1 support a C-terminal location for the MHC class II binding epitopes (33, 34). The determination of the amino acids necessary for MHC class II binding may provide a rationale to obtain mAb-SEA conjugates with preserved T cell-activating properties but totally devoid of binding to MHC class II molecules.

Antibody heteroconjugates and hybrid mAbs reacting with tumor cells and epitopes involved in T-cell activation, including the CD3–TCR complex, CD2, or CD28, have been used to target T cells to kill tumor cells *in vitro* (24–26, 35, 36). However, bispecific mAbs have several limitations as therapeutic agents: (*i*) the ability to directly bind to the T cells will ultimately lead to capture of the intravenously administered mAb in peripheral blood and perturb tissue penetration, (*ii*) binding to T cells in the absence of proper cross-linking by the target cell may lead to anergy (37) or cell death (38), and (*iii*) activation of an excessive number of T cells by pan-T-cell heteroconjugates may result in a cytokine-related shock syndrome and suppression of specific immunity dealing with infectious pathogens (39). In contrast, mAb-SEA conjugates do not suffer from these limitations. The ability of the conjugate to efficiently interact with T cells only when bound to the tumor cell surface allows effective tissue penetration and avoids unwanted systemic T-cell activation. Moreover, local production of lymphokines by T cells activated in the tumor area may be expected to result in a beneficial inflammatory response with direct effects on the tumor cells as well as recruitment of new anti-tumor effector cells in a cascade fashion. SEA is an extremely efficient inducer of interleukin 2, tumor necrosis factor, and γ interferon (14, 40). The activation of a limited fraction of T cells bearing the proper TCR V_β sequences preserves a large portion of the T-cell repertoire for dealing with specific immunity. Recently attempts to target antigen-specific $CD4^+$ T cells against tumor cells have been made with conjugates of mAbs and recall antigens such as keyhole limpet hemocyanin (41) and purified protein derivative (42). These mAb–antigen conjugates allowed presentation of relevant processed antigenic peptide fragments on MHC class II molecules and retargeting of CTLs to the tumor cells (41, 42). The recruited T cells are primarily of the CD4 type, and their frequency is significantly lower than those responding to SEA. Moreover, the therapeutic use of such mAb–antigen conjugates is limited to MHC class II^+ tumor cells, and, in contrast to the mAb-SEA conjugates, they do not have a general application for treating MHC class II^- tumors.

The mAb-SEA conjugates described in this study represent a novel class of anti-tumor agents based on conjugation of a superantigen to a tumor-reactive mAb. Development of superantigen–mAb-based agents may serve as an important immunotherapeutic strategy for treatment of malignant diseases that have escaped the host immune response.

We thank Ms. I. Andersson, B. Benediktsson, M. Borg, P. Karlström, M. Lassen, M. Mastej, Å. Norrman. C. Pettersson, and M. Tuneskog for excellent technical assistance.

1. Marrack, P. & Kappler, J. (1990) *Science* 248, 705–711.
2. Marrack, P., Kuschnir, E. & Kappler, J. (1991) *Nature (London)* 349, 524–526.
3. Frankel, W. N., Rudy, C., Coffin, J. M. & Huber, B. T. (1991) *Nature (London)* 349, 526–528.
4. Fisher, H., Dohlsten, M., Lindvall, M., Sjögren, H.-O. & Carlsson, R. (1989) *J. Immunol.* 142, 3151–3157.
5. Fraser, J. D. (1989) *Nature (London)* 339, 221–223.
6. Mollick, J. A., Cook, R. G. & Rich, R. R. (1989) *Science* 244, 817–820.
7. Fleischer, B. & Schrezenmeier, H. (1988) *J. Exp. Med.* 167, 1697–1707.
8. White, J., Herman, A., Pullen, A. M., Kubo, R., Kappler, J. & Marrack, P. (1989) *Cell* 56, 27–35.
9. Herman, A., Croteau, G., Sekaly, R.-P., Kappler, J. & Marrack, P. (1990) *J. Exp. Med.* 172, 709–717.
10. Scholl, P., Diez, A., Karr, R., Sekaly, R. P., Trowsdale, J. & Geha, R. (1990) *J. Immunol.* 144, 226–230.
11. Herrmann, T., Accolla, R. S. & MacDonald, H. R. (1989) *Eur. J. Immunol.* 19, 2171–2174.
12. Karp, D. R., Teletski, C. L., Scholl, P., Geha, R. & Long, E. O. (1990) *Nature (London)* 346, 474–476.
13. Dellabona, P., Peccoud, J., Kappler, J., Marrack, P., Benoist, C. & Mathis, D. (1990) *Cell* 52, 1115–1121.
14. Carlsson, R. & Sjögren, H. O. (1985) *Cell. Immunol.* 96, 175–182.
15. Dohlsten, M., Lando, P. A., Hedlund, G., Trowsdale, J. & Kalland, T. (1990) *Immunology* 1, 96–100.
16. Hedlund, G., Dohlsten, M., Lando, P. A. & Kalland, T. (1990) *Cell. Immunol.* 129, 426–434.
17. Herrman, T., Maryansky, J. L., Romero, P., Fleischer, B. & MacDonald, H. H. (1990) *J. Immunol.* 144, 1181–1186.
18. Dohlsten, M., Hedlund, G. & Kalland, T. (1991) *Immunol. Today* 12, 147–150.
19. Dohlsten, M., Hedlund, G., Lando, P. A., Trowsdale, J., Altmann, D., Patarroyo, M., Fischer, H. & Kalland, T. (1991) *Eur. J. Immunol.* 21, 131–135.
20. Foon, K. (1989) *Cancer Res.* 49, 1621–1639.
21. Pastan, I., Willingham, M. C. & Fitzgerald, D. J. (1986) *Cell* 47, 641–648.
22. Starling, J. J., Maciak, R. S., Hinson, A., Nichols, C. L., Briggs, S. L. & Laguzza, B. C. (1989) *Cancer Immunol. Immunother.* 28, 171–178.
23. Reiter, Y. & Fishelson, Z. J. (1989) *Immunology* 142, 2771–2777.
24. Perez, P., Hoffman, R. W., Shaw, S., Bluestone, J. A. & Segal, D. M. (1985) *Nature (London)* 316, 354–356.
25. Staerz, U. D., Kanagawa, O. & Bevan, M. J. (1985) *Nature (London)* 314, 628–631.
26. Jung, G., Ledbetter, J. A. & Müller-Eberhard, H. J. (1987) *Proc. Natl. Acad. Sci. USA* 84, 4611–4615.
27. Dohlsten, M., Hedlund, G., Segrén, S., Lando, P. A., Herrman, T., Kelley, A. & Kalland, T. (1991) *Eur. J. Immunol.* 21, 1229–1233.
28. Åkerblom, E. & Agback, H. (1990) Swed. Patent Appl. 9,002,490-2.
29. Carlsson, J., Drevin, H. & Axén, R. (1978) *Biochem. J.* 173, 723–737.
30. Wölfel, T., Klehmann, E., Muller, C., Schutt, K.-H., Meyer zum Buschenfelde, K.-H. & Knuth, A. (1989) *J. Exp. Med.* 170, 797–810.
31. Alexander, J., Rayman, P., Edinger, M., Conelly, R., Tubbs, R., Bukowsky, R., Pontes, E. & Finke, J. (1990) *Int. J. Cancer* 45, 119–124.
32. Fleischer, B., Gerardy-Schahn, R., Metzroth, B., Carrel, S., Gerlach, D. & Köhler, W. (1991) *J. Immunol.* 146, 11–17.
33. Blanco, L., Choi, E. M., Conolly, K., Thompson, M. R. & Bonventre, P. F. (1990) *Infect. Immun.* 58, 3020–3028.
34. Bohach, G. A., Handley, J. P. & Schlievert, P. M. (1989) *Infect. Immun.* 57, 23–28.
35. Jung, G. & Müller-Eberhard, H. J. (1988) *Immunol. Today* 9, 257–260.
36. Scott, C. F., Jr., Lambert, J. M., Kalish, R. S., Morimoto, C. & Schlossman, S. F. (1988) *J. Immunol.* 140, 8–14.
37. Schwartz, R. H. (1990) *Science* 248, 1349–1356.
38. Liu, Y. & Janeway, C. A., Jr. (1990) *J. Exp. Med.* 172, 1735–1739.
39. Ferran, C., Shehan, K., Dy, M., Schrieber, R., Merite, S., Landais, P., Noel, L.-H., Grau, G., Bluestone, J., Bach, J.-F. & Chatenoud, L. (1990) *Eur. J. Immunol.* 20, 509–515.
40. Fischer, H., Dohlsten, M., Andersson, U., Hedlund, G., Ericsson, P.-O., Hansson, J. & Sjögren, H.-O. (1990) *J. Immunol.* 144, 4663–4669.
41. Gravelle, A. & Ochi, A. J. (1989) *J. Immunol.* 142, 4079–4084.
42. Montgomery, A. M. P., Wing, M. G. & Lachmann, P. J. (1990) *Clin. Exp. Immunol.* 82, 200–207.